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SPLANCHNIC UPTAKE OF GLUCOSE, INORGANIC PHOSPHORUS, AND POTASSIUM AFTER AN INTRAVENOUS GLUCOSE LOAD IN PATIENTS WITH CIRRHOSIS AND IN SUBJECTS WITHOUT LIVER DISEASE

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STUDIES of the hepatic mechanism for blood glucose regulation in the intact organism have been carried out by Soskin and co-workers[1] in dogs and Bondy and co-workers[2] and Myers[3] in humans. These investigations revealed a hepatic secretion of glucose in the fasting state and a net uptake of blood glucose by the liver after glucose loading. In other studies, a concomitant net decrease in the serum concentration of inorganic phosphorus and potassium after glucose loading has been frequently recorded. Since these ions are known to be intimately related to glucose metabolism and hepatic glycogen synthesis,[4] we have studied the net changes in the concentration of inorganic phosphorus and potassium in blood entering (arterial) and leaving (hepatic venous) the liver and have attempted to relate these changes temporally to splanchnic glucose metabolism. These measurements were undertaken in a group of subjects without liver disease and in patients with *well-compensated* cirrhosis before and after the administration of intravenous glucose.

The arterial–hepatic venous (A-HV) differences reported here demonstrate the *net* resultant of changes in all of the splanchnic organs for the constituents

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TABLE I. PERTINENT DETAILS OF PATIENTS' HISTORY AND FINDINGS

PATIENT	SEX	AGE	WT. (KG.)	DIAGNOSIS	LIVER BIOPSY	SERUM BILIRUBIN (MG.%)	BSP* (%)	ALKALINE PHOSPHATASE† (K-A UNITS)	ZINC TURBIDITY‡ (UNITS)	THYMOL TURBIDITY (UNITS)	SERUM ALBUMIN (GM.%)	SERUM GLOBULIN (GM.%)
L. S.	M	52	70.0	Laennec's cirrhosis	Yes	0.8	16	12	5.0	0.5	4.3	3.0
R. J.	M	46	65.5	Laennec's cirrhosis	Yes	0.3	10	16	10.0	8.0	4.4	3.6
E. M.	M	41	76.3	Laennec's cirrhosis	Yes	0.6	20	8	6.0	4.0	3.1	4.2
T. W.	M	45	65.0	Laennec's cirrhosis	Yes	0.3	<10	4	2.0	2.0	5.0	2.7
J. M.	F	40	47.8	Laennec's cirrhosis	No	2.4		16	14.0	12.0	2.4	5.3
W. M.	M	15	58.2	Laennec's cirrhosis	No	1.6	28	68	9.0	8.0	2.8	4.3
P. H.	M	43	67.7	Laennec's cirrhosis	No	2.5		9.0	4.0	6.0	2.1	3.7
J. W.	M	60	58.2	Laennec's cirrhosis	Yes	1.0		12.0	14.0	11.0	2.6	5.4
R. B.	M	33	71.0	Laennec's cirrhosis	Yes	0.9	28	61	6.5	7.0	4.0	4.0
R. C.	F	40	52.8	Primary biliary cirrhosis	Yes	4.3		101	5.5	4.0	3.2	4.5
D. B.	M	50	68.7	Cor pulmonale	Yes	0.5	<10	9.0	4.0	3.0	4.2	3.6
J. D.	M	58	71.8	Splenomegaly	Yes	0.6	<10	2.0	5.0	1.5	3.6	3.7
M. C.	M	25	61.0	Extrahepatic portal block	Yes	0.4	<10	9.0	5.0	2.0	3.1	3.5
F. S.	M	52	61.3	Extrahepatic portal block	Yes	0.7	<10	7.0	4.0	1.0	4.4	3.3
B. M.	F	65	58.2	Riedel's lobe	No	0.3	<10	14.0	1.0	1.0	3.3	2.7
A. P.	F	50	57.3	Cholelithiasis splenomegaly	Yes	2.1		11.0	3.0	3.0	3.5	2.9

*Normal value: 0 to 10 per cent retention at 30 minutes.
†Normal value: 1.5 to 8.0 King-Armstrong units.
‡Normal value: 4 to 12 units.

measured, although it is likely that the changes largely reflect those of hepatic metabolism. These changes are independent of the possible variations in splanchnic blood flow during the observation period. Thus, in the present study, emphasis has been placed on the recording of changes in the direction of A-HV differences after glucose administration, rather than changes in magnitude.

Significant differences were observed between the patients with cirrhosis and control subjects for some of the parameters studied. The findings are discussed in terms of the known altered splanchnic blood flow in patients with cirrhosis and in the light of known potassium, inorganic phosphorus, and glucose interrelationships in vivo and in vitro.

MATERIALS AND METHODS

The data were obtained from patients who required cardiac catheterization for investigation of pathologic conditions of the heart or hepatic vein catheterization for assessment of portal hypertension. A summary of the pertinent clinical and chemical findings and the diagnosis for each of the patients is presented in Table I. Certain rigid criteria were satisfied for each subject before inclusion in the present series: (1) oral daily intake of carbohydrate greater than 250 Gm., (2) freedom from ascites and edema, (3) absence of a family history of diabetes mellitus, (4) absence of renal disease, (5) normal fasting blood sugar, and (6) administration of oral or parenteral vitamin B therapy for several days prior to investigation of the patients with cirrhosis.

All subjects had been fasting for 12 to 14 hours and were in the basal state; no premedication was administered. Under local anesthesia (Xylocaine without epinephrine), the cardiac catheter was inserted into an antecubital vein and directed under fluoroscopic control into the appropriate area. A slow infusion of 0.85 per cent saline containing 1,000 U. heparin per 250 ml. was used to maintain patency of the catheter. After the diagnostic procedures were completed, the catheter was placed so that the tip remained free in the hepatic vein. An indwelling needle was then fixed in the brachial artery of the opposite arm, and control blood samples were taken simultaneously from the hepatic vein and brachial artery. After 10 minutes, a second pair of control samples were drawn. Fifty milliliters of a 50 per cent solution of glucose was then injected in a 1 to 2 minute period into a superficial arm vein. After termination of glucose administration, hepatic venous and arterial blood was withdrawn simultaneously at 15, 30, 45, 60, 90, and 120 minutes for chemical determinations.

Immediately after withdrawal, the blood was transferred to containers with heparin, and the plasma was separated by centrifugation. If the chemical determinations were not completed on the day of the observation, the plasma was stored at −20° C. Freezing and storage in this manner did not affect the concentrations of the substances measured. Plasma glucose was determined by the Nelson method,[5] inorganic phosphorus by the Fiske and Subbarow method[6] and potassium by a standard flame photometric method. Glucose and inorganic phosphorus were determined in duplicate and potassium in quadruplicate.

RESULTS

Splanchnic Glucose Metabolism.—There was no significant difference in the mean arterial plasma glucose concentrations in the fasting state or at any interval after the intravenous glucose load between the 6 control subjects and the 10 patients with cirrhosis (Table II). In the fasting state, there was a net splanchnic glucose output in all patients. The mean, fasting, A-HV glucose difference in the patients with cirrhosis was less than in the control subjects; the difference, however, does not reach a significant level. Because one of the

TABLE II. CONCENTRATION OF GLUCOSE (MG. %) IN THE HEPATIC VENOUS AND PERIPHERAL ARTERIAL BLOOD PLASMA AFTER ADMINISTRATION OF INTRAVENOUS GLUCOSE TO PATIENTS WITH AND WITHOUT LIVER DISEASE

	ARTERIAL (MINUTES AFTER GLUCOSE)							HEPATIC VEIN (MINUTES AFTER GLUCOSE)						
PATIENTS	INITIAL*	15	30	45	60	90	120	INITIAL*	15	30	45	60	90	120
Patients With Cirrhosis.—														
L. S.	99	277	246	220	200	159	118	100	279	252	222	190	157	116
R. J.	100	264	228		194	159	116	112		224	210	182	144	128
E. M.	73	224	187	176	152	116	90	78	216	184	170	134	110	93
T. W.	85	252	216	176	162	130	99	96	256	207	180	172	131	102
J. M.	81	294	228	184	158	93	86	86	304	235	182	156	97	84
W. M.	68	205	166	129	101	74	78	72	203	152	109	84	88	94
P. H.	80	227	180	175	133	90	71	116	222	174	161	137	90	70
J. W.	70	224	168	153	117	70	62	81	222	152	127	101	71	65
R. B.	85	194	174	134	110	85	79	86	193	157	132	108	84	77
R. C.	106	278	213	190	143	111	88	112	304	227	192	163	123	102
Mean	85	244	201	171	147	109	89	94	244	196	168	143	110	93
S. E.	4	11	9	9	10	10	6	5	4	12	12	11	9	6
Control Subjects.—														
D. B.	93	256	224	201	182	132	107	114	261	232	202	181	130	104
J. D.	86	213	165	140	136	107	89	104	211	168	143	132	94	85
M. C.	86	217	168	114	91	62	72	102	224	164	110	84	76	98
F. S.	84	234	192	180	155	115	82	92	226	204	169	151	117	85
B. M.	68	237	202	180	163	127	96	79	248	201	167	158	119	90
A. P.	81	279	257	214	193	128	98	91	286	236	212	192	128	99
Mean	83	239	201	172	153	112	91	97	243	201	168	150	111	94
S. E.	3	10	14	15	15	11	5	5	11	12	15	16	9	3

*Mean value of 2 samples drawn before glucose administration.

TABLE III. CONCENTRATION OF INORGANIC PHOSPHORUS (MG. %) IN THE HEPATIC VENOUS AND PERIPHERAL ARTERIAL BLOOD PLASMA BEFORE AND AFTER ADMINISTRATION OF INTRAVENOUS GLUCOSE TO PATIENTS WITH AND WITHOUT LIVER DISEASE

	ARTERIAL (MINUTES AFTER GLUCOSE)							HEPATIC VEIN (MINUTES AFTER GLUCOSE)						
PATIENTS	INITIAL*	15	30	45	60	90	120	INITIAL*	15	30	45	60	90	120
Patients With Cirrhosis.—														
L. S.	2.67	2.64	2.57	2.60	2.55	2.28	2.38	2.64	2.32	2.36	2.41	2.30	2.34	2.36
R. J.	2.90	2.94	2.88	2.62	2.74	2.85	3.26	2.82	2.86	2.64	2.46	2.42	2.94	2.94
E. M.	3.82	3.69	3.36	3.14	3.14	3.54	3.72	3.88	3.40	3.22	3.28	3.18	3.43	3.73
T. W.	3.47	3.28	3.39	3.36	3.20	3.30	3.39	3.52	3.32	3.26	3.30	2.96	3.41	3.75
J. M.	3.18	3.03	2.85	2.76	2.82	2.80	3.08	3.24	2.88	2.48	2.63	2.70	2.88	3.05
W. M.	4.16	3.82	3.67	3.21	3.41	3.40	3.47	4.03	3.78	3.66	3.34	3.41	3.41	3.44
P. H.	3.22	3.07	2.82	2.78	2.66	2.63	2.98	3.15	3.04	2.77	2.60	2.76	2.63	2.96
J. W.	3.40	3.15	2.70	2.44	2.95	3.14	3.14	3.42	2.97	2.70	2.56	3.13	3.02	3.14
R. B.	3.40	3.28	3.14	2.99	3.09	3.34	3.45	3.59	3.23	3.25	3.07	3.16	3.35	3.52
R. C.	2.86	2.63	2.68	2.64	2.58	2.50	2.72	2.96	2.62	2.37	2.46	2.42	2.66	2.52
Mean	3.31	3.15	3.01	2.85	2.91	2.98	3.16	3.32	3.04	2.87	2.81	2.84	3.01	3.14
S. E.	0.14	0.12	0.12	0.09	0.09	0.14	0.12	0.14	0.13	0.14	0.12	0.12	0.12	0.15
Control Subjects.—														
D. B.	2.68	2.52	2.53	2.62	2.72	2.75	2.94	2.83	2.61	2.56	2.62	2.78	2.84	3.03
J. D.	3.24	2.98	2.99	2.94	2.97	3.07	3.18	3.14	3.15	2.80	2.77	2.75	3.04	3.18
M. C.	3.40	2.98	2.72	2.84	2.93	3.12	3.23	3.39	3.18	2.83	2.89		3.08	3.41
F. S.	2.81	2.61	2.56	2.48	2.52	2.49	2.55	2.65	2.64	2.48	2.46	2.48	2.42	2.54
B. M.	3.77	3.84	3.65	3.30	3.46	3.30	3.18	3.88	3.74	3.50	3.46	3.47	3.14	3.23
A. P.	3.66	3.54	3.07	3.26	3.38	3.80	3.77	3.57	3.43	3.18	3.24	3.53	3.55	3.98
Mean	3.26	3.08	2.92	2.91	3.00	3.09	3.14	3.24	3.12	2.89	2.91	3.00	3.01	3.23
S. E.	0.18	0.21	0.17	0.14	0.15	0.19	0.16	0.19	0.18	0.16	0.15	0.21	0.15	0.19

*Mean value of 2 samples drawn before glucose administration.

fasting values (P. H.) was more than four times the mean for the cirrhotic group, this value is suspect. If one omits this value from the statistical analysis, the difference between the two groups is highly significant ($p < 0.001$).

After the rapid intravenous glucose load, there was a marked variation between individuals for the time when a net splanchnic glucose uptake first became apparent. In only 3 of the subjects (E. M., P. H., and F. S.) was there a definite net splanchnic glucose uptake by 15 minutes after glucose administration; in a single patient with the diagnosis of primary biliary cirrhosis (R. C.), a net splanchnic glucose uptake was not recorded at any of the sampling intervals. The mean A-HV glucose differences did not vary significantly between the two groups of patients after glucose administration (Fig. 1).

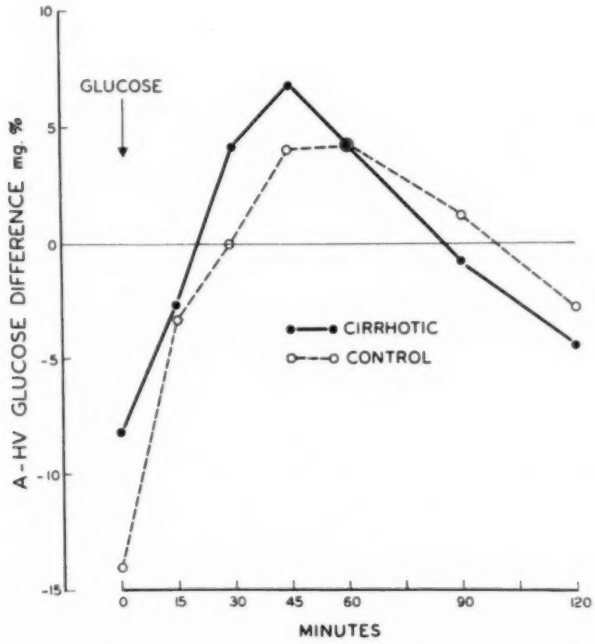

Fig. 1.—The plasma A-HV difference for glucose in the fasting state and after the intravenous administration of glucose.

Splanchnic Inorganic Phosphorus Metabolism.—After the intravenous administration of glucose, a decrease in the concentration of inorganic phosphorus in the arterial blood plasma occurred in both groups of patients (Table III). In the fasting state, the concentrations of inorganic phosphorus in the arterial and hepatic venous blood plasma were the same (Fig. 2). In view of the wide variation in individual responses, the data were analyzed by the multiple classification analysis of variance.[7] By this technique, a net splanchnic inorganic phosphorus uptake after glucose administration for the subjects with cirrhosis was significant at a level of $p < 0.01$ ($F = 12.99$ at 1 and 9 degrees of freedom). No significant splanchnic uptake of inorganic phosphorus could be demonstrated for the control subjects when the data were analyzed by the

same statistical technique. The mean net splanchnic uptake of inorganic phosphorus in the subjects with cirrhosis took place within the first hour after glucose administration.

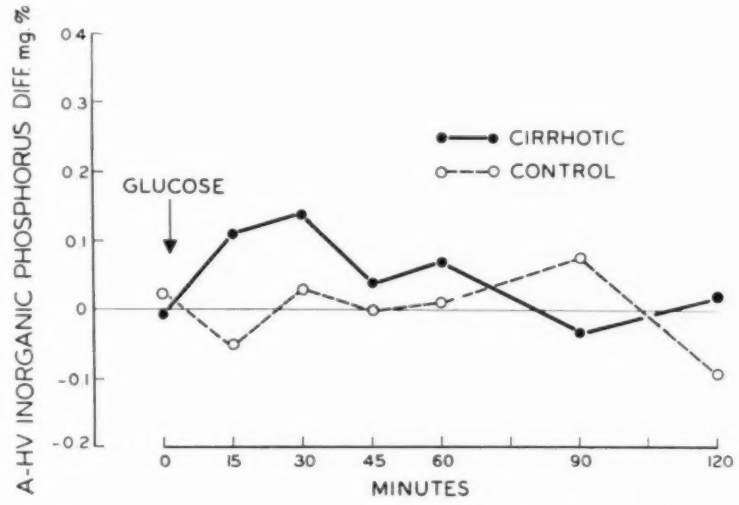

Fig. 2.—The plasma A-HV difference for inorganic phosphorus in the fasting state and after the intravenous administration of glucose.

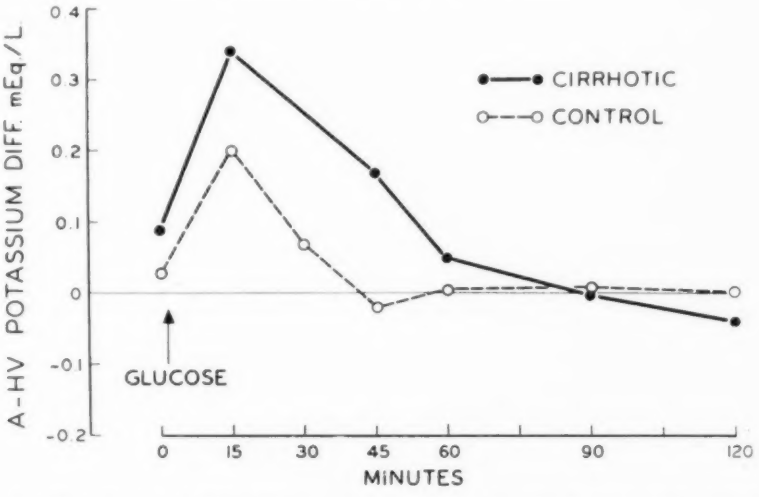

Fig. 3.—The plasma A-HV difference for potassium in the fasting state and after the intravenous administration of glucose. The 30 minute value for the patients with cirrhosis is omitted because of incomplete data.

Splanchnic Potassium Metabolism.—The arterial plasma potassium levels diminished in both groups of patients after glucose administration (Table IV). The arterial and hepatic venous concentrations of potassium did not differ significantly in the fasting state in either group of patients (Table IV). Although the mean A-HV values for both groups of patients are positive after

TABLE IV. CONCENTRATION OF POTASSIUM (mEq./L.) IN THE HEPATIC VENOUS AND PERIPHERAL ARTERIAL BLOOD PLASMA BEFORE AND AFTER ADMINISTRATION OF INTRAVENOUS GLUCOSE TO PATIENTS WITH AND WITHOUT LIVER DISEASE

PATIENTS	ARTERIAL (MINUTES AFTER GLUCOSE)							HEPATIC VEIN (MINUTES AFTER GLUCOSE)						
	INITIAL*	15	30	45	60	90	120	INITIAL*	15	30	45	60	90	120
Patients With Cirrhosis.—														
L. S.	4.15	4.05	3.84	3.70	3.70	3.58	3.55	3.86	3.85	3.82	3.62	3.69	3.69	3.74
R. J.	4.14	4.05	3.84	3.70	3.70	3.58	3.55	3.86	3.85	3.82	3.62	3.69	3.69	3.74
E. M.	4.00	3.55	3.46	3.64	3.50	3.54	3.52	3.82	3.27	3.48	3.35	3.55	3.55	3.62
T. W.	4.32	4.17	4.10	4.33	4.15	4.25	4.33	4.27	3.49		3.37	4.13	4.13	
J. M.	2.86	3.00	2.73	2.79	2.92	2.88	3.00	2.90	2.75	2.68	2.68	3.11	2.80	
W. M.	4.15	4.04	3.97	3.76	3.75	3.67	3.86	4.11	3.67	3.89	3.72	3.78	3.62	3.89
P. H.	4.70	4.64	4.58	4.46	4.50	4.49	4.43	4.76	4.30	4.64	4.52	4.40	4.46	4.45
J. W.	4.34	4.20	4.02	4.07	3.94	4.04	4.01	4.34	4.04	3.98	4.02	3.93	3.98	4.06
R. B.	4.30	4.11		3.93	3.98	3.95	4.10	4.18	3.91	4.09	4.18	3.89	4.08	4.08
R. C.	3.76	3.70	3.10	3.50	3.48	3.45	3.42	3.72	2.97		3.07	2.93	3.18	3.18
Mean	4.07	3.95	3.74	3.79	3.76	3.74	3.78	3.98	3.61	3.80	3.62	3.71	3.72	3.84
S. E.	0.16	0.14	0.19	0.15	0.14	0.14	0.14	0.16	0.16	0.20	0.17	0.14	0.15	0.13
Control Subjects.—														
D. B.	3.90	4.00	4.15	3.91	3.90	3.91	4.00	4.20	4.15	4.05	4.26	4.25	4.30	4.20
J. D.	3.71	3.71	3.48	3.19	3.05	3.61	3.38	3.40	3.29	3.35	2.90	3.00	2.95	3.34
M. C.	4.22	4.16	4.09	4.15	4.16	4.19	4.23	4.14	4.08	4.10	4.04	3.95	4.23	4.26
F. S.	3.90	3.62	3.62	3.78	3.65	3.48	3.59	3.83	3.32	3.52	3.69	3.67	3.56	3.58
B. M.	3.83	3.45	3.68	3.62	3.87	3.87	3.86	3.84	3.18	3.55	3.81	3.82	3.96	3.74
A. P.	3.99	3.77	3.78	3.95	4.06	3.93	3.84	3.94	3.50	3.82	4.01	3.88	3.96	3.84
Mean	3.92	3.78	3.80	3.77	3.78	3.83	3.82	3.89	3.59	3.73	3.78	3.76	3.83	3.83
S. E.	0.07	0.11	0.11	0.14	0.16	0.10	0.12	0.12	0.17	0.12	0.20	0.17	0.21	0.15

*Mean value of 2 samples drawn before glucose administration.

glucose administration (Fig. 3), an analysis of variance reveals a significant net splanchnic uptake ($p=0.005$, $F=13.29$ at 1 and 9 degrees of freedom) only in the patients with cirrhosis. The mean net splanchnic uptake of potassium in the cirrhotic group was greatest 15 minutes after glucose administration and had returned to the fasting level at 60 minutes.

DISCUSSION

The observations reported here confirm the finding of a splanchnic glucose output in the fasting state and a net splanchnic glucose uptake after glucose administration. Although an effect of the induced hyperglycemia on splanchnic glucose metabolism was noted as early as 15 minutes after glucose administration (Fig. 1), in approximately half the patients (L. S., T. W., J. M., D. B., B. M., A. P., and M. C.), cessation of splanchnic glucose output did not occur until 30 to 60 minutes had elapsed. In the comparable hepatic vein catheterization studies of Bondy and associates,[2] a net splanchnic glucose uptake was seen in all subjects at 15 minutes after the onset of glucose administration. This discrepancy may be attributable to the rate of sugar administration; in the present study glucose was rapidly injected intravenously, whereas in the earlier experiments the glucose was administered over a 15 minute period. Our data are consistent with those of Bondy and co-workers[2] in that a splanchnic glucose output may be apparent 90 to 120 minutes after glucose administration (Table II) at a time when the arterial glucose concentration is still elevated above the fasting level. The in vivo observations of Bearn and collaborators[8] on the decreased splanchnic glucose output after insulin administration suggest that endogenous insulin secretion, as a result of hyperglycemia, might influence hepatic glucose secretion.

After a glucose load, there is a fall in the peripheral blood inorganic phosphorus concentration. Some of the loss of inorganic phosphorus from the intravascular compartment can be accounted for by an increased urinary excretion of inorganic phosphorus.[9, 10] In other studies of large numbers of cirrhotic patients, it has been shown that the mean fall in blood inorganic phosphorus is greater than in normal subjects,[11, 12] but the urinary excretion is no greater in patients with cirrhosis than in normal subjects.[13] Our experiments suggest that there is also an intracellular uptake of inorganic phosphorus after glucose loading, and that the state of hepatic carbohydrate metabolism influences this uptake. On the basis of intracellular measurements of phosphorus in the livers of rabbits given cortisone, Nichols[14] has postulated that inorganic phosphorus is taken up by muscle rather than by liver after glucose loading. However, the direct measurement of inorganic phosphorus across the splanchnic organs, reported here, clearly indicates a net splanchnic uptake of inorganic phosphorus after a glucose load in patients with cirrhosis. A net splanchnic uptake of inorganic phosphorus could not be demonstrated in the control subjects under these conditions. The difference between the patients with cirrhosis and control subjects in this respect may be explained in two ways, either: (1) splanchnic inorganic phosphorus metabolism differs

in the two groups under the conditions of these observations or (2) the cirrhotic liver compensates for a decreased blood flow by extracting more inorganic phosphorus per milliliter of blood than does the normal liver. If the latter hypothesis were true, one would not expect to be able to detect the small inorganic phosphorus A-HV differences across the normal liver.

Glucose loading in the human results in a movement of potassium out of the intravascular compartment with a consequent fall in serum potassium levels. Groen and co-workers[15] have shown that there is no extra urinary potassium loss after glucose administration, and these authors postulate that the potassium enters the cells in exchange for hydrogen ion to form the potassium salts of the metabolic products of glucose catabolism. The influence of potassium on hepatic glycogen deposition in vitro has been studied extensively by Hastings and co-workers.[4] In addition, potassium has been shown to be an essential cofactor for the anaerobic metabolism of glucose.[16] Our data indicate that there was a significant net uptake of potassium by the splanchnic organs after glucose administration in the subjects with cirrhosis but not in the control patients. As was the case with splanchnic inorganic phosphorus metabolism, these results may mean that splanchnic potassium metabolism differs in the two groups of subjects, or that the cirrhotic liver extracts more potassium from the blood after glucose loading than does the control liver. The latter interpretation implies that there is a net splanchnic uptake of potassium after glucose loading in the patients with normal livers, but the A-HV differences are too small to measure accurately.

In the fasting subjects with cirrhosis, the negative A-HV glucose difference was significantly less than in the control subjects. This finding, together with the fact that splanchnic blood flow is decreased in cirrhosis[17] implies reduction in the absolute amount of glucose released by the liver. Although Myers[3] found a decreased hepatic glucose output in cirrhosis, a difference in the A-HV difference between normal subjects and cirrhotic patients was not observed. The decreased hepatic glucose output in cirrhosis is probably due to reduction of glycogen stores, and a decreased rate of glucose production from precursors.[18] Despite the reduced hepatic output of glucose in the fasting patient with cirrhosis, the peripheral glucose concentration remains at normal levels. This must mean that peripheral utilization is decreased, or that there is an appreciable extrahepatic source of glucose or that both conditions exist. Decreased peripheral utilization is suggested by the data of Lang and associates,[19] who demonstrated impaired peripheral glucose utilization in eviscerated dogs. There is much evidence, obtained from animal studies, to support the alternative hypothesis of an extrahepatic source of blood glucose. The kidneys have been shown to secrete glucose into the blood in the eviscerated rat,[20, 21] in the eviscerated or hepatectomized rabbit,[22] in hepatectomized or eviscerated dogs,[23, 24] and in the anesthetized[25] and unanesthetized intact dog.[26]

SUMMARY

Observations of the net splanchnic metabolism of glucose, inorganic phosphorus, and potassium by means of simultaneous sampling of arterial and

hepatic venous blood have been made in 10 patients with *well-compensated* cirrhosis and 6 control subjects in the fasting state and after rapid intravenous glucose administration. Emphasis was placed on determination of the direction rather than the magnitude of change of the parameters studied.

1. A net splanchnic glucose output was noted in both groups of patients during the fasting state. The arterial, fasting blood sugar was normal in the subjects with cirrhosis despite a decreased splanchnic glucose output.

2. After glucose administration there was a net splanchnic uptake of glucose. In only about half the cases was the uptake first recorded between 30 and 60 minutes after glucose administration. No differences could be detected between the patients with cirrhosis and control subjects.

3. There was a significant net splanchnic uptake of inorganic phosphorus after glucose administration in the patients with cirrhosis but not in the control subjects.

4. A mean net splanchnic potassium uptake was noted in both groups of patients, but the arterial–hepatic venous differences were significant only in the subjects with cirrhosis.

We would like to acknowledge the excellent nursing assistance of Sister Mary Cooke during the catheterization procedure. Mr. N. W. Please of University College, London, and Dr. John Fertig and colleagues of Columbia University assisted with statistical analysis of the data.

REFERENCES

1. Soskin, S., Essex, H. E., Herrick, J. F., and Mann, F. C.: The Mechanism of Regulation of the Blood Sugar by the Liver, Am. J. Physiol. **124**: 558, 1938.
2. Bondy, P. K., James, D. F., and Farrar, B. W.: Studies of the Role of the Liver in Human Carbohydrate Metabolism by the Venous Catheter Technic. I. Normal Subjects Under Fasting Conditions and Following the Injection of Glucose, J. Clin. Invest. **28**: 238, 1949.
3. Myers, J. D.: Net Splanchnic Glucose Production in Normal Man and in Various Disease States, J. Clin. Invest. **29**: 1421, 1950.
4. Renold, A. E., Ashmore, J., and Hastings, A. B.: Regulation of Carbohydrate Metabolism in Isolated Tissues, Vitamins & Hormones **14**: 139, 1956.
5. Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose, J. Biol. Chem. **153**: 375, 1944.
6. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375, 1925.
7. Snedecor, G. W.: Statistical Methods, ed. 5, Ames, Iowa, 1956, The Iowa State College Press.
8. Bearn, A. G., Billing, B. H., and Sherlock, S.: Response of the Liver to Insulin; Hepatic Vein Catheter Studies in Man, *in* Wolstenholme, G. E. W., editor: Ciba Foundation Colloquia on Endocrinology. Vol. 6, Hormonal Factors in Carbohydrate Metabolism, Boston, 1953, Little, Brown & Company, p. 250.
9. Levitan, B. A.: Effect in Normal Man of Hyperglycemia and Glycosuria on Excretion and Reabsorption of Phosphate, J. Appl. Physiol. **4**: 224, 1951.
10. Huffman, E. R., Hlad, C. J., Jr., Whipple, N. E., and Elrick, H.: The Influence of Blood Glucose on the Renal Clearance of Phosphate, J. Clin. Invest. **37**: 369, 1958.
11. Smith, L. H., Jr., Ettinger, R. H., and Seligson, D.: A Comparison of the Metabolism of Fructose and Glucose in Hepatic Disease and Diabetes Mellitus, J. Clin. Invest. **32**: 273, 1953.
12. Danowski, T. S., Gillespie, H. K., Fergus, E. B., and Puntereri, A. J.: Significance of Blood Sugar and Serum Electrolytes Changes in Cirrhosis Following Glucose, Insulin, Glucagon, or Epinephrine, Yale J. Biol. & Med. **29**: 361, 1956.
13. Hoenig, V., Schück, O., Fischer, O., Hoenigová, J., and Patová, V.: Hyperphosphaturie après administration intraveineuse de glucose et de fructose, Acta med. scandinav. **161**: 79, 1958.

14. Nichols, N.: The Effect of Glycogen Deposition on Liver Phosphorus, J. Clin. Invest. **34**: 1710, 1955.
15. Groen, J., Willebrands, A. F., Kamminga, C. E., Van Schothorst, H. K., and Godfried, E. G.: Effects of Glucose Administration on the Potassium and Inorganic Phosphate Content of the Blood Serum and the Electrocardiogram in Normal Individuals and in Non-diabetic Patients, Acta med. scandinav. **141**: 352, 1952.
16. Stumpf, P. K.: Glycolysis, *in* Greenberg, D. M., editor: Chemical Pathways of Metabolism, New York, 1954, Academic Press, Inc., vol. 1, p. 67.
17. Bradley, S. E., Ingelfinger, F. J., Groff, A. E., and Bradley, G. P.: Estimated Hepatic Blood Flow and Hepatic Venous Oxygen Content in Cirrhosis of the Liver, Proc. Soc. Exper. Biol. & Med. **67**: 206, 1948.
18. Bondy, P. K.: Some Metabolic Abnormalities in Liver Disease, Am. J. Med. **24**: 428, 1958.
19. Lang, S., Goldstein, M. S., and Levine, R.: Influence of Liver on Uptake of Glucose by Extrahepatic Tissues, Am. J. Physiol. **177**: 447, 1954.
20. Roberts, S., and Samuels, L. T.: Fasting and Gluconeogenesis in the Kidney of the Eviscerated Rat, Am. J. Physiol. **142**: 240, 1944.
21. Reinecke, R. M.: The Kidney as a Source of Glucose in the Eviscerated Rat, Am. J. Physiol. **140**: 276, 1943.
22. Bergman, H., and Drury, D. R.: The Relationship of Kidney Function to the Glucose Utilization of the Extra Abdominal Tissues, Am. J. Physiol. **124**: 279, 1938.
23. Reinecke, R. M., and Hauser, P. J.: Renal Glucogenesis in the Eviscerated Dog, Am. J. Physiol. **153**: 205, 1948.
24. Cohn, C., and Kolinsky, M.: Effect of Blood Sugar Levels and Insulin Lack on Gluconeogenesis by the Kidney of the Dog, Am. J. Physiol. **156**: 345, 1949.
25. Cohn, C., Katz, B., and Kolinsky, M.: Renal Glucogenesis in the Intact Dog, Am. J. Physiol. **165**: 423, 1951.
26. McCann, W. P., and Jude, J. R.: The Synthesis of Glucose by the Kidney, Bull. Johns Hopkins Hosp. **103**: 77, 1958.

EFFECT OF INTRAVENOUS GLUCOSE ON SPLANCHNIC AND PERIPHERAL METABOLISM OF ENDOGENOUS PYRUVATE AND CITRATE IN PATIENTS WITH CIRRHOSIS AND IN SUBJECTS WITHOUT LIVER DISEASE

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IN THE previous communication, the hepatic vein catheterization technique was applied to the study of splanchnic glucose, inorganic phosphorus, and potassium metabolism.[1] In a further attempt to define the splanchnic metabolism of glucose, changes in the concentrations of two key intermediates of carbohydrate metabolism, pyruvate and citrate, were measured in blood drawn simultaneously from the hepatic vein and a peripheral artery after glucose administration. These observations were made in both patients with cirrhosis and in subjects without liver disease. The changes in splanchnic pyruvate and citrate metabolism are discussed in terms of the known pathways of intermediary carbohydrate metabolism.

In other observations, peripheral arterial-venous pyruvate and citrate differences were measured in order to assess the pyruvate and citrate contributed by the peripheral tissues to the blood.

MATERIALS AND METHODS

The subjects studied and the method of the hepatic vein catheterization were those reported previously[1]; one additional control patient (H. M.) was studied. When peripheral arterial-venous (A-V) differences for citrate and pyruvate were measured at the time of hepatic vein catheterization, peripheral venous blood samples were drawn through an indwelling needle in a superficial arm vein or in the femoral vein. In one subject without liver disease, peripheral A-V differences were obtained under the same conditions but without hepatic vein catheterization.

Blood for the determination of α-keto acids was collected at 0° C., and one volume of blood was added to two volumes of cold 20 per cent trichloroacetic acid within 2 minutes of sampling. The supernatant was then immediately treated as described by Friedemann and Haugen.[2] This method specifically measures the pyruvic and α-ketoglutaric acids of blood. In studies of fasting, normal subjects[3, 4] and patients with cirrhosis,[5-7] the blood pyruvic to α-ketoglutaric acid ratio has been found to be between 5 to 1 and 10 to 1 by chromatographic analysis. After glucose administration, peripheral blood levels of pyruvate and α-ketoglutarate increase in both groups, but the ratio remains approximately the same.[8] It has been shown[9] that after the addition of base to equimolar concentrations of the 2-4, dinitrophenylhydrazones of these two α-keto acids, absorption at 360 to 400 mμ by the pyruvic derivative is about four times that of the α-ketoglutaric derivative. For these

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reasons, our results are expressed in terms of pyruvic acid. Plasma citric acid determinations were carried out by the Elliott modification of the Natelson, Pincus, and Lugovoy method.[10] All determinations were done in duplicate.

RESULTS

Splanchnic Pyruvate Metabolism.—The mean fasting arterial pyruvate level was slightly higher in the patients with cirrhosis than in the control subjects (Table I), but the difference between the two groups was not statistically significant. After a glucose load the arterial pyruvate concentration rose in all

TABLE I. CONCENTRATION OF PYRUVATE (μG/ML.) IN HEPATIC VENOUS AND ARTERIAL BLOOD BEFORE AND AFTER ADMINISTRATION OF INTRAVENOUS GLUCOSE TO PATIENTS WITH AND WITHOUT LIVER DISEASE

	ARTERIAL (MINUTES AFTER GLUCOSE)					HEPATIC VENOUS (MINUTES AFTER GLUCOSE)				
PATIENTS	INITIAL	30	60	90	120	INITIAL	30	60	90	120
Patients With Cirrhosis.—										
E. M.	10.6		13.0	14.3		7.9		13.4	16.1	
T. W.	9.8	13.3		9.3	8.2	9.5	17.6		9.6	6.8
J. M.	13.0	17.9	15.1	15.7	14.2	12.5	16.7	16.7	14.7	13.2
N. M.	14.6	18.2	15.6	14.6	14.1	13.1	17.9	15.8	13.2	12.5
P. H.	12.5	14.6	13.8	12.9	13.1	11.4	14.6	12.8	12.6	11.7
J. W.	10.0	12.8	12.6	12.2	12.4	9.6	13.1	13.8	13.9	13.1
Mean	11.8	15.4	14.0	13.2	12.4	10.7	16.0	14.5	13.4	11.5
S. E.	0.8	1.2	0.6	0.9	1.1	0.8	0.9	0.8	0.9	1.2
Control Subjects.—										
D. B.	11.1	13.4	14.0	12.6	11.5	10.6	17.4	17.8	14.7	13.8
M. C.	13.1	17.6	14.4	13.2	12.7	17.0	26.7	18.9	15.8	13.6
F. S.	10.5	11.2	11.8	10.3	10.2	10.0	10.0	9.7	10.3	9.9
B. M.	8.5	11.1	12.0	9.8	11.1	6.7	9.1	11.9	10.4	8.6
A. P.	10.2	13.6	13.8	10.8	10.3	9.3	13.9	13.9	10.9	9.0
Mean	10.7	13.4	13.2	11.3	11.2	10.7	15.4	14.4	12.4	11.0
S. E.	0.7	1.2	0.5	0.7	0.5	1.7	3.2	1.7	1.2	0.9

subjects; again, no significant difference was noted between the control subjects and patients with cirrhosis. In all but one subject (M. C.), the fasting arterial blood level was either the same or higher than the concentration in the hepatic vein. After a glucose load, a mean, negative arterial–hepatic venous (A–HV) difference was apparent at 30, 60, and 90 minutes in both patients with cirrhosis and control subjects, indicating a net splanchnic production of pyruvate (Fig. 1). At 120 minutes a net splanchnic pyruvate uptake was evident; this occurred at a time when the arterial glucose concentration had returned to the fasting level. There was no significant pyruvate A–HV difference between the patients with cirrhosis and the control subjects at any time interval after glucose administration.

Splanchnic Citrate Metabolism.—The mean, fasting, arterial blood plasma citrate concentration was slightly higher in the patients with cirrhosis as compared to the control subjects (Table II); however, the difference between the two groups was not statistically significant. In the fasting state a positive A–HV difference in both the patients with cirrhosis and control subjects indicates

TABLE II. CONCENTRATION OF CITRATE (μG/ML.) IN HEPATIC VENOUS AND ARTERIAL BLOOD PLASMA BEFORE AND AFTER ADMINISTRATION OF INTRAVENOUS GLUCOSE TO PATIENTS WITH AND WITHOUT LIVER DISEASE

	ARTERIAL (MINUTES AFTER GLUCOSE)							HEPATIC VENOUS (MINUTES AFTER GLUCOSE)						
PATIENTS	INITIAL	15	30	45	60	90	120	INITIAL	15	30	45	60	90	120
Patients With Cirrhosis.—														
L. S.	33.6	30.8	31.1	29.7	32.6	23.4	18.2	19.8	24.5	19.2	17.8	18.2	10.6	9.6
E. M.	21.4	19.8	19.5	18.7	15.8	15.8	18.9		17.0	18.3	12.6	16.8	10.4	11.8
J. M.	31.5	25.3	26.3	25.0	25.8	21.2	23.0	30.0	27.2	24.0	23.0	22.5	20.4	24.7
W. M.	43.4	41.6	44.0	41.1	38.6	38.4	39.4	39.8	40.7	35.5	35.7	32.2	30.0	31.6
P. H.	25.6	29.8	31.0	29.2	31.1	21.8	20.9	11.0	17.9	25.6	26.7	26.3	22.4	20.4
J. W.	20.7	19.1	18.9	13.5	8.7	8.4	11.1	18.4	19.6	16.5	10.2	9.2	10.9	7.3
R. C.	15.2	14.7	13.2	12.2	10.7	6.4	9.8	9.6	11.1	9.5	11.2	6.9	7.3	6.4
Mean	27.3	25.9	26.3	24.2	23.3	19.3	20.2	21.4	22.6	21.2	19.6	18.9	16.0	16.0
S. E.	3.6	3.4	3.9	3.9	4.4	4.0	3.7	4.7	3.6	3.1	3.6	3.4	3.2	3.7
Control Subjects.—														
J. D.	16.8	16.8	14.8	10.3	10.6	7.9	9.1	12.4	13.1	7.3	7.4	7.8	7.0	8.8
M. C.	17.0	15.6	15.6	13.3	13.2	14.7	9.8	8.7	6.6	10.4	7.2		11.0	6.2
F. S.	23.4	21.0	18.9	20.4	17.8	15.3	13.8	15.8	16.4	19.0	15.5	14.4	16.9	15.8
B. M.	19.8	20.6	23.0	24.7	20.1	19.6	18.4	20.4	17.7	22.1	20.0	21.8	18.1	12.0
A. P.	33.7	25.4	30.7	19.4	16.1	12.2	15.4	31.2	26.5	23.8	14.2	12.0	11.2	12.8
H. M.	29.4	26.8	27.7	27.0	27.4	27.8	29.8	25.3	24.0	20.5	23.8	22.4	24.8	24.8
Mean	23.4	21.0	21.8	19.2	17.5	16.2	16.0	19.0	17.4	17.2	14.7	15.7	14.8	13.4
S. E.	2.8	1.8	2.7	2.6	2.4	3.2	3.1	3.4	3.0	2.7	2.7	2.8	2.8	2.7

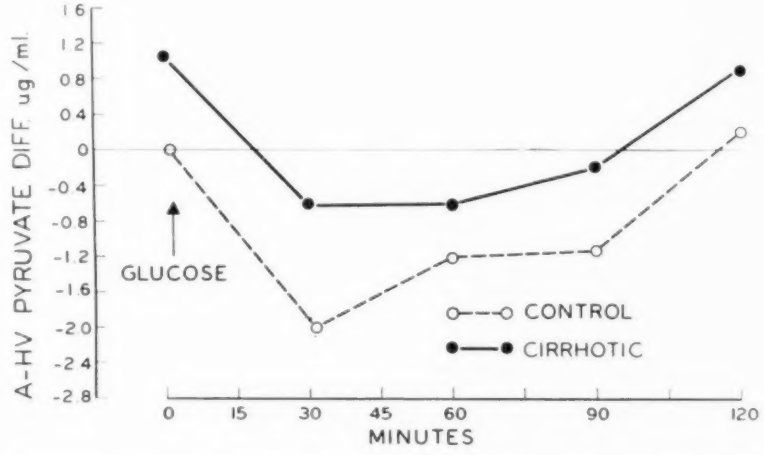

Fig. 1.—The whole blood A–HV difference for pyruvate in the fasting state and after the intravenous administration of glucose.

net splanchnic uptake of citrate* (Fig. 2). After intravenous glucose administration, the average arterial plasma citrate concentration fell slowly during the entire period of observation. A net splanchnic citrate uptake was evident at all sampling intervals. An analysis of variance using the multiple classification technique[13] revealed that the A–HV difference did not change after glucose administration ($F = 0.95$ at 36 and 6 degrees of freedom, $p > 0.25$), and that

*It has been shown that the concentration of citrate in the portal venous blood of rabbits, cats,[11] and dogs[12] is greater than in arterial blood; therefore it would be more accurate to use the term, *net hepatic uptake*, here.

the A–HV difference was not significantly different at any time interval between the 2 groups of patients ($F = 0.50$ at 1 and 11 degrees of freedom, $p > 0.25$).

Peripheral Pyruvate and Citrate Metabolism.—It can be seen from Fig. 3 that there is a net peripheral release of citrate into the venous blood as indicated by a negative A-V difference. After glucose administration, the peripheral A-V citrate difference narrows between 30 and 90 minutes and returns to fasting levels at 120 minutes. Although the data suggest that the fall in the arterial level of citrate after glucose administration was due to a decreased peripheral release of citrate, this interpretation does not take into account possible changes in peripheral blood flow. No A-V pyruvate differences were noted either in the fasting state or after glucose administration (Fig. 3).

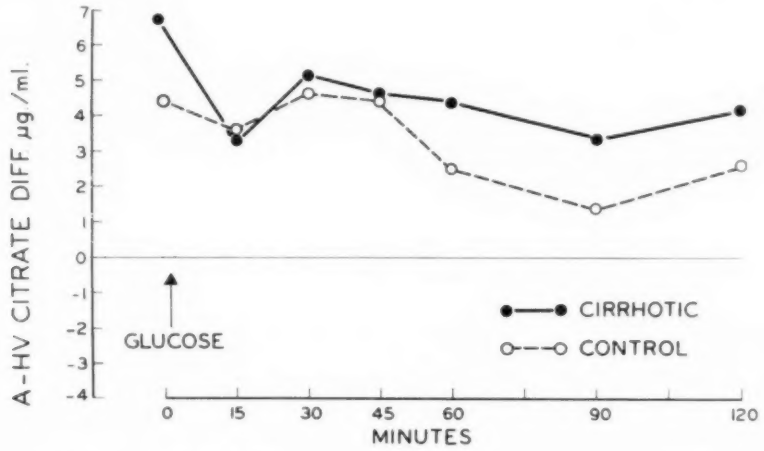

Fig. 2.—The plasma A–HV difference for citrate in the fasting state and after the intravenous administration of glucose.

DISCUSSION

The simultaneous measurements of pyruvate and citrate in blood entering and leaving the splanchnic area and the peripheral tissues provide a means of following the metabolic fate of injected glucose in humans. Our observations confirm previous studies[14] which demonstrated a small net splanchnic uptake of pyruvate under fasting, basal conditions. It is possible that pyruvate serves as one of the substrates for hepatic glucose formation under such conditions. In the investigations of others,[5, 6, 15] the concentration of pyruvate in the peripheral blood of subjects with cirrhosis exceeded that in the control subjects. The present study does not bear out this finding. The discrepancy is most likely due to the inclusion of only subjects with *well-compensated* cirrhosis in our study. The fasting A–HV pyruvate difference did not vary significantly between the two groups.

After glucose administration the splanchnic uptake of pyruvate ceases and there is a small but significant output of pyruvate by the splanchnic organs:

this occurs at a time when glucose is being utilized by the splanchnic organs. A–HV pyruvate differences after glucose administration did not vary significantly between the two groups. Apparently the tissues of the extremities do not markedly influence the blood pyruvate concentration under the conditions of these observations (Fig. 3).

Citrate is actively metabolized by the kidneys of intact dogs[17] and by perfused, isolated rabbit and cat livers.[11, 18] The data presented here indicate that the human liver is also capable of metabolizing appreciable amounts of citrate. Although the peripheral plasma citrate level has been reported to be elevated in liver disease,[19] no difference in the fasting arterial or A–HV levels could be demonstrated between patients with cirrhosis as compared with subjects free

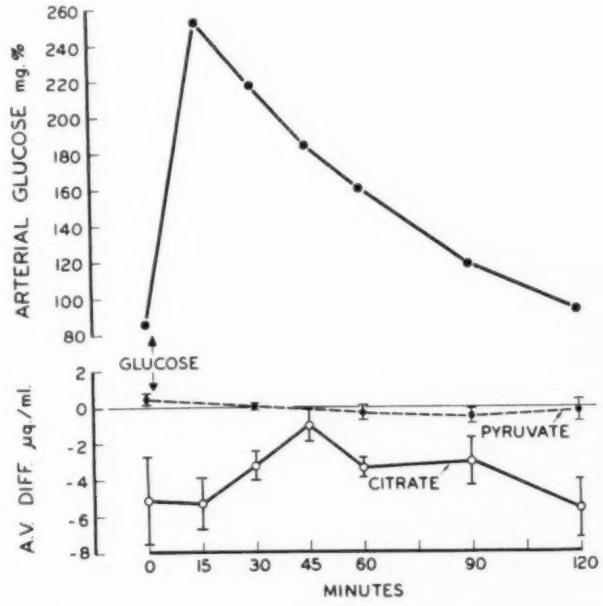

Fig. 3.—The peripheral A-V differences for pyruvate and citrate in the fasting state and after glucose administration. The values represent data obtained from 7 patients. The vertical lines indicate the mean ± standard error of the mean. The mean arterial glucose concentration is depicted at the top of the figure.

from liver disease in this study. Our data on the uptake of blood citrate by the liver and the data of Herndon and Freeman[17] on the renal uptake of citrate are consistent with the hypothesis that the citrate of the blood is turned over at a very rapid rate. The average clearance of citrate by the liver of subjects in a fasting state was 20 per cent (A–HV difference × 100 divided by arterial concentration). In the fasting state, maintenance of a relatively constant peripheral blood level in the face of rapid clearance from the blood is accomplished by the extrahepatic splanchnic organs[11, 12] and by the peripheral tissues[20] (Fig. 3). Thus, despite the low concentration of citrate in the peripheral blood (1.5 to 3.0 mg. per cent), its rapid turnover would suggest that it serves as an

important intermediate in the metabolism of the organism. Such a situation is analogous to the important metabolic role of the rapid turnover of unesterified fatty acids of the blood.[21]

In the experiments reported here, the level of citrate in the peripheral blood falls after a glucose load as has been reported by Natelson and co-workers,[22] yet the splanchnic uptake of citrate continues (Fig. 2).

Measurements of the concentration of citrate in the arterial and venous blood plasma of an extremity suggest that the peripheral citrate output diminishes after glucose administration (Fig. 3). In view of the demonstrations of large stores of citrate in bone by Dickens,[23] and the demonstration of citrate release from bone of intact dogs,[24] it seems likely that glucose must directly or indirectly influence the citrate release from bone rather than muscle.

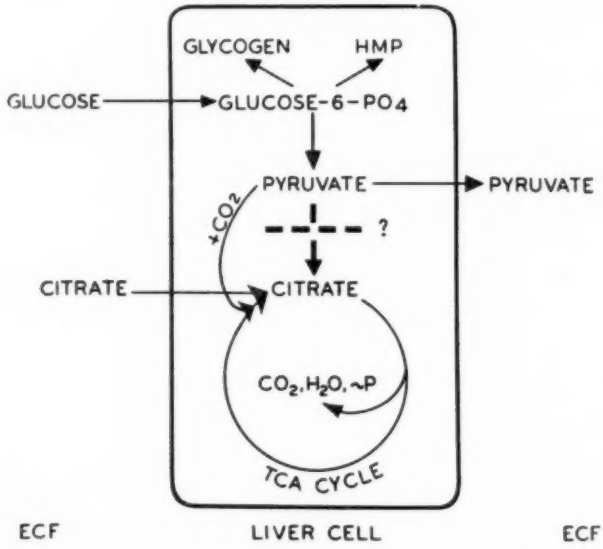

Fig. 4.—Schematic representation of intracellular hepatic metabolism. The heavy interrupted line indicates the postulated rate limiting mechanism after glucose administration, as discussed in the text. The arrows traversing the cell membrane indicate the direction of net change of the metabolites. The following abbreviations have been used: *ECF*, extracellular fluid, *HMP*, hexose monophosphate shunt, *~P*, adenosine triphosphate, and *TCA cycle*, tricarboxylic acid cycle.

The simultaneous splanchnic release of pyruvate and uptake of citrate after glucose loading is provided as evidence for the existence of a rate limiting mechanism in the metabolism of pyruvate to CO_2 and H_2O via acetyl CoA formation and subsequent condensation with oxaloacetate to yield citrate (Fig. 4). This interpretation receives support from the recent investigations of Freedman and Graff[25] with rat liver. These authors concluded from their studies with C^{14}-alanine in the fasted rat that the carbon atoms of pyruvate enter the citric acid cycle by means of CO_2 fixation. There are several possible explanations for these observations: (1) deficiency of enzymes or cofactors which are required to convert pyruvate to acetyl CoA, (2) deficiency in

the enzyme system responsible for the condensation of acetyl CoA with oxaloacetate, or (3) relative lack of acetyl CoA acceptor, i.e., oxaloacetate. The last possibility seems remote since the oxidation of citrate would provide oxaloacetate, and in addition oxaloacetate could be formed directly from pyruvate by CO_2 fixation.

SUMMARY

A study of subjects with *well-compensated* cirrhosis and patients without liver disease by means of the hepatic vein catheterization technique revealed a net splanchnic uptake of pyruvate and citrate from the blood in the basal state. After intravenous glucose administration there was a net splanchnic pyruvate production in the face of continuing splanchnic citrate uptake. No significant differences were noted between the two groups of patients.

Peripheral arterial-venous (A-V) differences demonstrated a release of citrate from the peripheral tissues in the fasting state. After glucose administration, the A-V citrate difference diminished. There was no difference between the arterial and peripheral venous pyruvate concentrations under the conditions of these observations.

The data are consistent with the hypotheses that the plasma citrate is rapidly turned over in the organism, and that a rate limiting mechanism is present in the liver for the conversion of pyruvate to CO_2 and H_2O via acetyl CoA and subsequent condensation of the latter compound with oxaloacetate to form citrate after glucose administration.

We would like to acknowledge the excellent nursing assistance of Sister Mary Cooke during the catheterization procedure. Mr. N. W. Please of University College, London, and Dr. John Fertig and colleagues of Columbia University assisted with the statistical analysis of the data.

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REFERENCES

1. Gordon, E. E., Craigie, A., Kerr, D. N. S., Cherrick, G. R., and Sherlock, S.: Splanchnic Uptake of Glucose, Inorganic Phosphorus, and Potassium After an Intravenous Glucose Load in Patients With Cirrhosis and in Subjects Without Liver Disease, J. Lab. & Clin. Med. **55**: 829, 1960.
2. Friedemann, T. E., and Haugen, G. E.: Pyruvic Acid. II. The Determination of Keto Acids in Blood and Urine, J. Biol. Chem. **147**: 415, 1943.
3. Seligson, D., and Shapiro, B.: Alpha Keto Acids in Blood and Urine Studied by Paper Chromatography, Anal. Chem. **24**: 754, 1952.
4. El Hawary, M. F. S., and Thompson, R. H. S.: Separation and Estimation of Blood Keto Acids by Paper Chromatography, Biochem. J. **53**: 340, 1953.
5. Seligson, D., McCormick, G. J., and Sborov, V.: Blood Ketoglutarate and Pyruvate in Liver Disease, J. Clin. Invest. **31**: 661, 1952. (Abstract.)
6. Dawson, A. M., de Groote, J., Rosenthal, W. S., and Sherlock, S.: Blood Pyruvic-Acid and Alpha-Ketoglutaric-Acid Levels in Liver Disease and Hepatic Coma, Lancet **1**: 392, 1957.
7. Summerskill, W. H. J., Wolfe, S. J., and Davidson, C. S.: The Metabolism of Ammonia and Alpha-Keto-Acids in Liver Disease and Hepatic Coma, J. Clin. Invest. **36**: 361, 1957.
8. Smith, L. H., Jr., Ettinger, R. H., and Seligson, D.: A Comparison of the Metabolism of Fructose and Glucose in Hepatic Disease and Diabetes Mellitus, J. Clin. Invest. **32**: 273, 1953.
9. Long, C.: A General Method for the Estimation of Alpha-Keto Acids, and Its Application to Alpha-Keto-Acid Metabolism in Pigeon Brain, Biochem. J. **36**: 807, 1942.

10. Stern, J. R.: Assay of Tricarboxylic Acids, *in* Colowick, S. P., and Kaplan, N. O., editors: Methods in Enzymology, New York, 1957, Academic Press, Inc., vol. 3, p. 425.
11. Mårtensson, J.: On the Citric Acid Metabolism in Mammals, Acta physiol. scandinav. (supp. 2) **1: 1,** 1940.
12. Harrison, H. C., and Harrison, H. E.: Interrelation of Calcium and Citrate Metabolism, Fed. Proc. **17:** 66, 1958.
13. Snedecor, G. W.: Statistical Methods, ed. 5, Ames, Iowa, 1956, The Iowa State College Press.
14. Craig, J. W., Drucker, W. R., Miller, M., Owens, J. E., Woodward, H., Jr., Brofman, B., and Pritchard, W. H.: Metabolism of Fructose by the Liver of Diabetic and Nondiabetic Subjects, Proc. Soc. Exper. Biol. & Med. **78:** 698, 1951.
15. Amatuzio, D. S., and Nesbitt, S.: A Study of Pyruvic Acid in the Blood and Spinal Fluid of Patients With Liver Disease With and Without Hepatic Coma, J. Clin. Invest. **29:** 1486, 1950.
16. Bradley, S. E., Ingelfinger, F. J., Groff, A. E., and Bradley, G. P.: Estimated Hepatic Blood Flow and Hepatic Venous Oxygen Content in Cirrhosis of the Liver, Proc. Soc. Exper. Biol. & Med. **67:** 206, 1948.
17. Herndon, R. F., and Freeman, S.: Renal Citric Acid Utilization in the Dog, Am. J. Physiol. **192:** 369, 1958.
18. Sjöström, P.: Der Citratgehalt im Blutserum als Diagnosticum bei Krankheiten der Leben und der Gallenwege, Acta chir. scandinav. (supp. 49) **79:** 1, 1937.
19. Thunberg, T.: Occurrence and Significance of Citric Acid in the Animal Organism, Physiol. Rev. **33:** 1, 1953.
20. Axelrod, D. R.: Relationship of Calcium and Citric Acid to Hyperventilation in Man, J. Clin. Invest. **36:** 870, 1957. (Abstract.)
21. Frederickson, D. S., and Gordon, R. S., Jr.: The Metabolism of Albumin Bound C^{14}-labeled Unesterified Fatty Acids in Normal Human Subjects, J. Clin. Invest. **37:** 1504, 1958.
22. Natelson, S., Pincus, J. B., and Lugovoy, J. K.: Response of Citric Acid Levels to Oral Administration of Glucose. I. Normal Adults and Children, J. Clin. Invest. **27:** 446, 1948.
23. Dickens, F.: The Citric Acid Content of Animal Tissues With Reference to Its Occurrence in Bone and Tumour, Biochem. J. **35:** 1011, 1941.
24. Neuman, W. F., Firschein, H., Chen, P. S., Jr., Mulryan, B. J., and DiStefano, V.: On the Mechanism of Action of Parathormone, J. Am. Chem. Soc. **78:** 3863, 1956.
25. Freedman, A. D., and Graff, S.: The Metabolism of Pyruvate in the Tricarboxylic Acid Cycle, J. Biol. Chem. **233:** 292, 1958.

THE EFFECT OF ACUTE HYPERVENTILATION ON THE BLOOD AMMONIA CONCENTRATION OF PATIENTS WITH LIVER DISEASE

The Relative Role of the Kidney, Liver, and Extremity

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PATIENTS with liver disease may exhibit progressive increases in arterial ammonia concentration in the apparent absence of an exogenous ammonia source.[1, 2] In these instances, the kidney, liver, brain, and extremities represent potential contributors to the blood ammonia.[3] Previous observations have demonstrated that the kidney usually releases ammonia into the systemic circulation,[4-6] whereas the other organs concerned with ammonia metabolism generally serve as ammonia receivers.[7-9] The parenteral administration of acetazoleamide[10] and chlorothiazide[11] to patients with liver disease results in a prompt increase in the release of ammonia into the renal vein, which is associated with a progressive rise in the arterial ammonia concentration. The magnitude of this increased increment of ammonia released into the renal vein after administration of acetazoleamide and chlorothiazide may be correlated with the extent of the rise in urine pH. Hyperventilation may also produce an alkaline urine,[12] and, in addition, may alter organ ammonia removal through changes in the $NH_3 : NH_4$ ratio[13] and the pH gradient between blood and tissue.[14] In view of these reports, as well as the hyperpnea and respiratory alkalosis[2] exhibited by patients with liver disease and encephalopathy, observations have been made on the effect of acute induced hyperventilation on the arterial ammonia concentration.

METHODS

Twenty men with liver disease were studied. Their ages ranged from 27 to 73 years. Liver biopsy revealed Laennec's cirrhosis in 13 patients, fatty metamorphosis in 2, and hepatitis in 1. Three were clinically cirrhotic, and 1 had chemical dysfunction. None displayed evidence of precoma or progressive hepatic deterioration at the time of the study.

All patients were recumbent and in the fasting state. Arterial samples were obtained from a brachial artery, and renal (13 patients) and hepatic (7 patients) venous samples by means of a No. 8 intravenous catheter previously positioned under fluoroscopic control. Renal venous blood was identified from catheter position and apparent arterialization; a narrowed arterial-venous oxygen difference was regarded as confirmatory. Femoral venous blood was obtained from an indwelling needle in 5 of the 7 patients who had undergone hepatic vein catheterization.

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Simultaneous arterial and venous samples were obtained during 1 or 2 control periods. Each patient was then instructed to hyperventilate continuously for 20 minutes. Samples were collected after 5 to 10 minutes of hyperventilation, after 20 minutes of hyperventilation, and 20 minutes after hyperventilation had ceased.

Blood ammonia (NH_3) was measured by a modification[8] of the microdiffusion method of Brown and co-workers.[15] The pH of whole blood was measured at room temperature with a Cambridge model R pH meter equipped with an enclosed glass electrode; the results were corrected to 37° C. by Rosenthal's factor.[16] The carbon dioxide content of whole blood was determined by the method of Van Slyke and Neill.[17] Carbon dioxide tension was calculated from this value, the pH and hemoglobin concentration by the line chart of Van Slyke and Sendroy,[18] using a pK of 6.11. Oxygen content and saturation were determined by the spectrophotometric method of Hickam and Frayser.[19]

In this study, a positive arterial-venous ammonia difference across an organ was interpreted as ammonia uptake, whereas a negative arterial-venous ammonia difference indicated ammonia release.

RESULTS

Hyperventilation was associated with a rise in arterial pH and a fall in pCO_2 in all patients. Mean values were as follows: resting pH = 7.41 ± 0.05, pCO_2 = 38.2 ± 7.2 mm. Hg; after 5 to 10 minutes of hyperventilation, pH = 7.57 ± 0.08, pCO_2 = 23.9 ± 6.1 mm. Hg; after 20 minutes of hyperventilation, pH = 7.60 ± 0.09, pCO_2 = 22.6 ± 6.5 mm. Hg; and 20 minutes after hyperventilation had ceased pH = 7.45 ± 0.07 and pCO_2 34 ± 7.0 mm. Hg.

The Effect of Hyperventilation on the Arterial Ammonia Concentration.—As shown in Table I, arterial ammonia concentration increased significantly during the first 10 minutes of hyperventilation and remained elevated during

TABLE I. THE EFFECT OF VOLUNTARY HYPERVENTILATION ON THE ARTERIAL AMMONIA CONCENTRATION* IN PATIENTS WITH LIVER DISEASE

PATIENT	CONTROL	DURING HYPERVENTILATION		20 MIN. AFTER HYPERVENTILATION
		5 TO 10 MIN.	20 MIN.	
J. F.	78	90	99	—
J. C.	91	99	104	100
W. N.	229	207	238	214
M. W.	87	105	113	95
T. I.	76	80	73	70
A. H.	71	99	97	—
S. J.	113	166	169	161
M. Wil.	84	88	101	94
C. F.	123	133	134	151
V. W.	121	127	153	142
W. Na.	134	149	141	160
J. B.	127	182	204	160
Mil. W.	105	115	144	145
H. P.	120	108	135	172
C. W.	84	99	88	80
A. W.	114	135	130	113
L. W.	107	114	130	125
E. L.	90	113	131	102
C. K.	111	119	108	110
M. C.	130	129	129	141
Mean	110	123	131	130
p†		<0.01	<0.01	<0.01

*Micrograms per 100 ml. (NH_3). Upper limit of normal for this laboratory is 108 μg per 100 ml.

†Refers to change from control value.

the remainder of the study, including the 20 minute posthyperventilation period. None of the patients exhibited evidence of neurologic deterioration during or after the period of overbreathing.

The Effect of Hyperventilation on Renal Ammonia Release.—Resting arterial-venous ammonia differences were consistently negative (Fig. 1). (Mean = −41 ± 29 μg per 100 ml., indicating release of ammonia into the systemic circulation.) There was considerable individual variation during both the hyperventilation and recovery periods, and mean arterial-renal venous differences remained essentially unchanged from those of control values. However, the release of ammonia into the renal vein after 20 minutes of hyperventilation was significantly decreased when compared with values obtained after 5 to 10 minutes (mean = −29 and −44 μg per 100 ml., respectively, $p = < 0.05$).

Ammonia Uptake by Liver and Lower Extremity.—Resting values for hepatic and femoral arterial-venous ammonia differences were consistently positive, indicating ammonia removal by the liver and periphery (Fig. 1). Although the mean arterial-venous ammonia differences across both liver and lower extremity increased during hyperventilation, significance was approached ($p = < 0.1$) only by the values obtained across the liver after 20 minutes of hyperventilation.

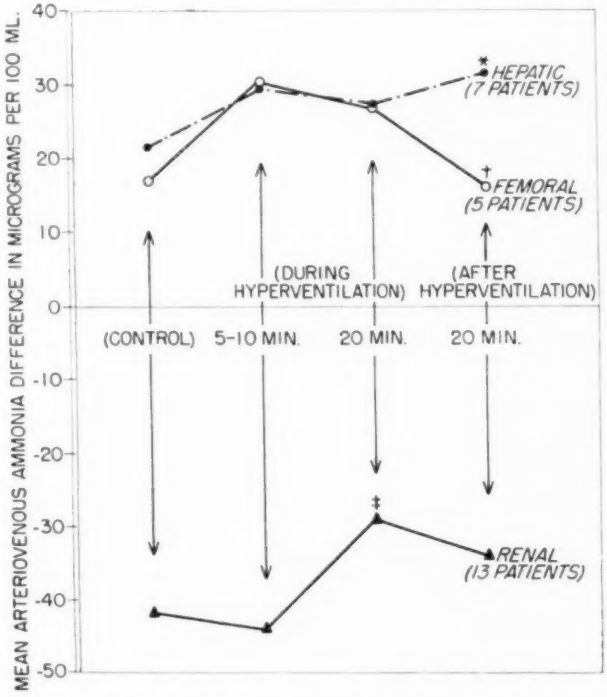

Fig. 1.—The effect of voluntary hyperventilation on the mean hepatic, femoral, and renal arterial-venous ammonia difference in patients with liver disease. *, Significant ($p = < 0.05$) as compared with baseline value. †, Significant ($p = < 0.02$) as compared with 20 minute hyperventilation period. ‡, Significant ($p = < 0.05$) as compared with 5 minute hyperventilation period.

During the recovery period, the mean hepatic arterial-venous ammonia difference was significantly greater ($p = < 0.05$) than the control value.

DISCUSSION

It is apparent that patients with liver disease with or without arterial hyperammonemia may exhibit significant elevations in arterial ammonia concentration during and after acute voluntary hyperventilation. The origin of this increased increment of blood ammonia could be attributed either to a greater endogenous ammonia production or to a decrease in organ removal.

Renal arterial-venous ammonia differences were variably affected by hyperventilation, and the kidney did not appear to be a source of the observed increase in arterial ammonia concentration. Indeed, there was a modest but significant decrease in the release of ammonia into the renal vein when values obtained during the initial phase of hyperventilation were compared with the 20 minute period, a time when the arterial concentration was maximally increased. Renal blood flow,[10] measured concomitantly in 8 patients, was not significantly altered. Previous observations in patients with liver disease given acetazoleamide[10] and chlorothiazide[11] intravenously have suggested that the relative amounts of ammonia released into the urine and renal vein are dependent upon their respective pH's; acetazoleamide and chlorothiazide rapidly and uniformly increase urine pH and decrease ammonia release into the urine, whereas arterial and renal venous pH remain essentially unchanged and renal vein ammonia increases twofold. Voluntary hyperventilation in normal subjects usually results in a rise in urine pH and a decrease in urine ammonia excretion which is somewhat variable in its temporal relationship.[12] In contrast to acetazoleamide and chlorothiazide, hyperventilation is associated with a rapid and fairly uniform increase in arterial pH and, presumably, renal venous pH. The observed individual variations in the release of ammonia into the renal vein during hyperventilation are in keeping with a more complex effect than that seen after administration of acetazoleamide or chlorothiazide since both tubular urine and renal venous pH are probably variably altered by acute respiratory alkalosis. Simultaneous measurements of urine and renal venous blood should be of aid in this evaluation.

Although release of ammonia from the liver[5] and extremity[1, 9] has been reported in some patients with marked hepatic dysfunction and encephalopathy, the present data indicate that acute respiratory alkalosis does not alter the primary role of these organs as ammonia receivers. Despite the absence of significant changes in arterial-venous ammonia difference for both organs, the general trend was that of increased uptake. The observations across the periphery are consistent with recent studies,[14] which have shown that 60 to 90 minutes of respiratory alkalosis results in an increased muscle ammonia concentration in hyperammonemic dogs. The lack of significant differences at comparable pH's, however, suggests that time may be a major factor or that other physiologic variants may be in effect in these patients with chronic liver disease. The significant increase in arterial-hepatic venous difference at the

20 minute posthyperventilation period is in contrast to the declining value for peripheral ammonia uptake. This difference in response may be explained by the several factors known to affect hepatic ammonia metabolism[3] other than the probable influence of a blood-tissue pH gradient.

It is apparent that the origin of the increased increment of blood ammonia in hyperventilated patients with liver disease remains speculative. Earlier studies[20] have demonstrated an elevated ammonia content in venous blood draining an exercising forearm. An increased ammonia concentration has been observed "during the hyperpneic period following exercise."[21] It would seem possible since muscle has an active glutamic dehydrogenase system[3] that the respiratory muscles may be a source of the observed increase in ammonia concentration.

SUMMARY

Voluntary hyperventilation in 20 patients with liver disease resulted in a significant elevation of arterial ammonia concentration which was evident at 10 and 20 minutes and persisted during a 20 minute posthyperventilation period.

The origin of this increased increment of blood ammonia could not be attributed to a greater release of ammonia into the renal vein, nor to a lessened uptake of ammonia by the liver or lower extremity. Each organ maintained its primary role as an ammonia donor (e.g., kidney) or ammonia receiver (e.g., liver and peripheral muscle) throughout the entire period of observation.

The possible sources of ammonia during acute hyperventilation are discussed.

REFERENCES

1. Summerskill, W. H., Wolfe, S. J., and Davidson, C. S.: The Metabolism of Ammonia and α-Keto-Acids in Liver Disease and Hepatic Coma, J. Clin. Invest. **36**: 361, 1957.
2. Tyor, M. P., and Sieker, H. O.: Biochemical, Blood Gas, and Peripheral Circulatory Alterations in Hepatic Coma, Am. J. Med. **27**: 50, 1959.
3. Bessman, S. P.: Ammonia Metabolism in Animals, in McElroy, W., and Glass, B.: Amino Acid Metabolism, Baltimore, 1955, Johns Hopkins Press, p. 408.
4. McDermott, W. V., Jr., Adams, R. D., and Riddell, A. G.: Ammonia Metabolism in Man, Ann. Surg. **140**: 539, 1954.
5. White, L. P., Phear, E. A., Summerskill, W. H. J., and Sherlock, S.: Ammonium Tolerance in Liver Disease; Observations Based on Catheterization of the Hepatic Vein, J. Clin. Invest. **34**: 158, 1955.
6. Berry, J. N., Flanagan, J. F., Owen, E. E., and Tyor, M. P.: The Kidney as a Source of Blood Ammonia in Resting and Hyperventilated Cirrhotics, Clin. Res. Proc. **7**: 154, 1959. (Abstract.)
7. Bessman, S. P., and Bessman, A. N.: The Cerebral and Peripheral Uptake of Ammonia in Liver Disease With an Hypothesis for the Mechanism of Hepatic Coma, J. Clin. Invest. **34**: 622, 1955.
8. Tyor, M. P., and Wilson, W. P.: Peripheral and Biochemical Changes Associated With the Intravenous Ammonium Salts in Normal Subjects, J. LAB. & CLIN. MED. **51**: 592, 1958.
9. Webster, L. T., Jr., and Gabuzda, G. J.: Ammonia Uptake by the Extremities and Brain in Hepatic Coma, J. Clin. Invest. **37**: 414, 1958.
10. Owen, E. E., Tyor, M. P., Flanagan, J. F., and Berry, J. N.: The Kidney as a Source of Blood Ammonia in Patients With Liver Disease: The Effect of Acetazoleamide, Clin. Res. Proc. **7**: 292, 1959. (Abstract.) J. Clin. Invest. In press Feb., 1960.
11. Owen, E. E., Flanagan, J. F., and Tyor, M. P.: The Kidney as a Source of Blood Ammonia in Patients With Liver Disease: The Effect of Chlorothiazide, Proc. Soc. Exper. Biol. & Med. **102**: 696, 1959.

12. Barker, E. S., Singer, R. B., Elkinton, J. R., and Clark, J. K.: The Renal Response in Man to Acute Experimental Respiratory Alkalosis and Acidosis, J. Clin. Invest. **36**: 515, 1957.
13. Warren, K. S.: The Differential Toxicity of Ammonium Salts, J. Clin. Invest. **37**: 497, 1958.
14. Stabenau, J. R., Warren, K. S., and Rall, D. P.: The Role of pH Gradient in the Distribution of Ammonia Between Blood and Cerebro-Spinal Fluid, Brain and Muscle, J. Clin. Invest. **38**: 373, 1959.
15. Brown, R. H., Duda, G. P., Korkes, S., and Handler, P.: A Colorimetric Micro-method for the Determination of Ammonia; the Ammonia Content of Rat Tissue and Human Plasma, Arch. Biochem. **66**: 301, 1957.
16. Rosenthal, T. B.: The Effect of Temperature on the pH of Blood and Plasma in Vitro, J. Biol. Chem. **173**: 25, 1948.
17. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements, J. Biol. Chem. **61**: 523, 1924.
18. Van Slyke, D. D., and Sendroy, J., Jr.: Studies of Gas and Electrolyte Equilibria in Blood. Line Charts for Graphic Calculations by the Henderson-Hasselbalch Equation and for Calculating Plasma Carbon Dioxide From Whole Blood Content, J. Biol. Chem. **79**: 781, 1928.
19. Hickam, J. B., and Frayser, R.: Spectrophotometric Determination of Blood Oxygen, J. Biol. Chem. **180**: 457, 1949.
20. Parnas, J. K., Mozolowski, W., and Lewinski, W.: Uber den Ammoniakgehalt und die Ammoniakbildung im Blute: Mitterlung der zusammen des Blutammoniaks mit der Muskelarbeit, Biochem. Ztschr. **188**: 15, 1927.
21. Schwartz, A. E., Lawrence, W., Jr., and Roberts, K. E.: Elevation of Peripheral Blood Ammonia Following Muscular Exercise, Proc. Soc. Exper. Biol. & Med. **98**: 548, 1958.

AMMONIA TOLERANCE IN LIVER DISEASE

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THE first ammonia tolerance tests were reported by Van Caulaert and Deviller[1] in 1932. These authors found no rise in venous blood ammonia after the ingestion of 5 Gm. of ammonium chloride in normal subjects or patients with obstructive jaundice or hepatitis. They noted, however, a rapid and sustained elevation of the blood ammonia concentration in patients with cirrhosis. The occurrence of normal ammonia tolerance in hepatitis, despite severe hepatic parenchymal damage, led them to conclude that abnormal ammonia tolerance in cirrhosis was due to the shunting of portal blood to the systemic circulation rather than to impaired extraction of ammonia by the liver. Kirk,[2] in similar studies, found no rise in the blood ammonia in patients with acute hepatitis or obstructive jaundice after 10 Gm. of ammonium citrate had been given, but he, too, noted abnormal ammonia tolerance in patients with cirrhosis. Moreover, he demonstrated that urea synthesis was normal in each of these groups and concurred with the conclusion that abnormal ammonia tolerance resulted from the diversion of portal blood through collateral veins to the systemic circulation. Roger and Stahl[3] also found abnormal ammonia tolerance tests in patients with cirrhosis or portal vein obstruction, and normal ammonia tolerance in patients with other diseases of the liver. In addition, these authors studied hepatic and peripheral venous ammonia levels in patients with cirrhosis given 5 Gm. of ammonium acetate and demonstrated impaired hepatic clearance of ammonia.[4] They postulated that the elevated hepatic vein ammonia levels resulted from inadequate contact between portal blood and the hepatic parenchymal cells caused by the architectural changes of cirrhosis, and concluded that the increased peripheral blood ammonia levels were due to both intra- and extrahepatic portasystemic anastomoses.[4, 5]

White and co-workers[6] recently reported a series of ammonia tolerance tests which was at variance with these previous investigations. After the administration of 3 Gm. of ammonium chloride they noted abnormal ammonia tolerance in patients with hepatitis and obstructive jaundice as well as in those with cirrhosis. Simultaneous measurement of hepatic vein ammonia levels led these authors to attribute the abnormal results to defective hepatocellular

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function in patients with hepatitis, and to both portasystemic shunting and decreased hepatic extraction in patients with cirrhosis. The abnormal tests in patients with obstructive jaundice could not be interpreted satisfactorily.

The explanation for these contradictory observations is not clear. The dosage, type of ammonium compounds administered, and method used for the determination of blood ammonia differed in each study (Table I). None of these investigators related in detail the clinical aspects of the patients to the ammonia tolerance test.

The present study was undertaken to reassess the ammonia tolerance test in patients with various types of liver disease and to correlate the test results with the clinical, laboratory, and histologic aspects of these disorders.

TABLE I. AMMONIA TOLERANCE TEST: TECHNIQUES EMPLOYED

INVESTIGATOR AND REFERENCE	AMMONIUM SALT	AMOUNT EMPLOYED (GM.)	CONTENT OF NH_3 N (MG.)	BLOOD NH_3 TECHNIQUE
Van Caulaert and Deviller[1]	Ammonium chloride	5	1,350	Parnas
Kirk[2]	Ammonium citrate	10	1,730	Van Slyke and Hiller
Roger and Stahl[3]	Ammonium acetate	5	1,100	Parnas
White and co-workers[6]	Ammonium chloride	3	790	Conway
Conn	Ammonium chloride	3	790	Seligson and Hirahara

METHODS

All observations were made on patients who had been admitted to the West Haven Veterans Administration Hospital or the Grace-New Haven Community Hospital. All patients had been fasting for 9 to 12 hours and none were receiving antibiotics, arginine, or glutamic acid at the time the test was performed. Tests were not carried out on patients with impending hepatic coma, severe nausea, or vomiting. Blood was withdrawn from an antecubital vein without stasis or exercise, after which Bromsulphalein dye was injected. Organon heparin (10 mg. per milliliter) was used for anticoagulation. Three grams of ammonium chloride was administered as nonenteric coated tablets with approximately 250 ml. of water. The patients remained at rest throughout the study. Forty-five and 120 minutes later blood was drawn from the opposite arm for blood ammonia and Bromsulphalein determinations. In some patients arterial blood was obtained through an indwelling Cournand needle simultaneously with the venous samples. Arterial pH was determined before and after ammonium chloride administration in some patients.

The blood ammonia concentrations were determined according to the method of Seligson and Hirahara.[7] The Bromsulphalein method of Gaebler[8] was utilized. Liver function tests were done according to the methods previously described from these laboratories[9] except that serum proteins were measured by the Gornall biuret technique.[10, 11] Hydrogen ion concentration was determined with the Cambridge model R pH meter. Serum glutamic oxalacetic transaminase (SGOT) was performed by the method of Reitman and Frankel.[12] The Vim Silverman needle was used for liver biopsies and the intercostal approach was employed. Esophagoscopic examinations were performed using the flexible Eder-Hufford esophagoscope.

RESULTS

The control group consisted of 18 normal volunteers and 7 patients with minor nonhepatic disorders. The mean fasting blood ammonia level in these subjects was 102 μg per 100 ml. and ranged from 60 to 150 μg per 100 ml.

Characteristically, the ammonia tolerance test in this group was flat (Fig. 1). The blood ammonia concentration 45 and 120 minutes after administration of ammonia chloride showed no significant change from that of the fasting levels (Table II). In no instance did the 45 minute blood ammonia concentration exceed the upper limit of normal (150 μg per 100 ml.). Bromsulphalein retention was normal in each subject.

Nineteen patients with obstructive jaundice of diverse etiologies were studied. In 17 the diagnosis was confirmed by biopsy, surgical exploration, or autopsy. In each of these patients the liver function tests were characterized by increased levels of serum bilirubin, alkaline phosphatase, and Bromsulphalein retention. The cephalin flocculation and thymol turbidity tests were invariably normal. With a single exception, all patients exhibited normal ammonia tolerance (Fig. 1).

TABLE II. AMMONIA TOLERANCE TESTS IN LIVER DISEASE

DIAGNOSIS	NO. IN GROUP	BLOOD AMMONIA* (μG/100 ML.)			BROMSULPHALEIN RETENTION* (%)
		FASTING	45 MIN.	120 MIN.	
Control subjects	25	102 ± 23	104 ± 24	97 ± 18	3 ± 1.8
Obstructive jaundice	19	103 ± 20	119 ± 25	110 ± 18	40 ± 20
Hepatitis	22	100 ± 20	142 ± 64	—	40 ± 18
Cirrhosis	60	132 ± 60	260 ± 60	209 ± 27	33 ± 15
Portacaval shunts	18	140 ± 45	342 ± 85	252 ± 52	26 ± 10
Hepatic fibrosis and/or fatty infiltration	15	100 ± 19	115 ± 17	112 ± 18	21 ± 13

*Mean ± standard deviation.

Twenty-two patients with hepatitis were studied. Seventeen had viral hepatitis and the remainder had drug induced hepatitis. Histologic confirmation was obtained in 20. Normal ammonia tolerance was observed in 16 (Fig. 1). The 6 patients with abnormal ammonia tolerance tests were studied serially and will be discussed below. Bromsulphalein retention was increased in all patients.

Sixty patients with cirrhosis were studied. The diagnosis was confirmed histologically in 50 and was evident clinically in the others. Fifty-two patients had Laennec's cirrhosis, 4 posthepatic cirrhosis, 3 hemochromatosis, and 1 cirrhosis secondary to sarcoidosis. Approximately half of the patients exhibited jaundice, ascites, and edema. The other half had less severe degrees of hepatic decompensation. Abnormal ammonia tolerance curves were observed in 50 patients. These abnormalities characteristically consisted of a high normal or elevated fasting blood ammonia level and a large sustained elevation after ammonium chloride (Fig. 1, Table II). Normal ammonia tolerance was exhibited by 10 patients.

In 18 patients with cirrhosis with portasystemic shunts, ammonia tolerance tests showed the mean fasting blood ammonia level was at the upper limit of normal. The 45 and 120 minute blood ammonia levels grossly exceeded the corresponding levels in the patients with cirrhosis (Fig. 1, Table II). The mean Bromsulphalein retention, however, was less abnormal than in the patients with cirrhosis.

Fatty infiltration of the liver, portal fibrosis, or both were diagnosed by liver biopsy in 15 patients. Although there was an increase in the fibrous tissue of the portal zones, there was no distortion of the normal lobular pattern. Ammonia tolerance tests were normal in all 15 patients.

Ammonia tolerance tests with simultaneous arterial and venous sampling were performed in control subjects and in patients with hepatitis or cirrhosis to evaluate the role of the peripheral tissues on ammonia tolerance (Fig. 2, Table III).* These studies in general confirmed the results obtained with venous determinations alone, but, in addition, demonstrated that occasional false normal tests occurred when based on venous standards alone. One of 4 patients

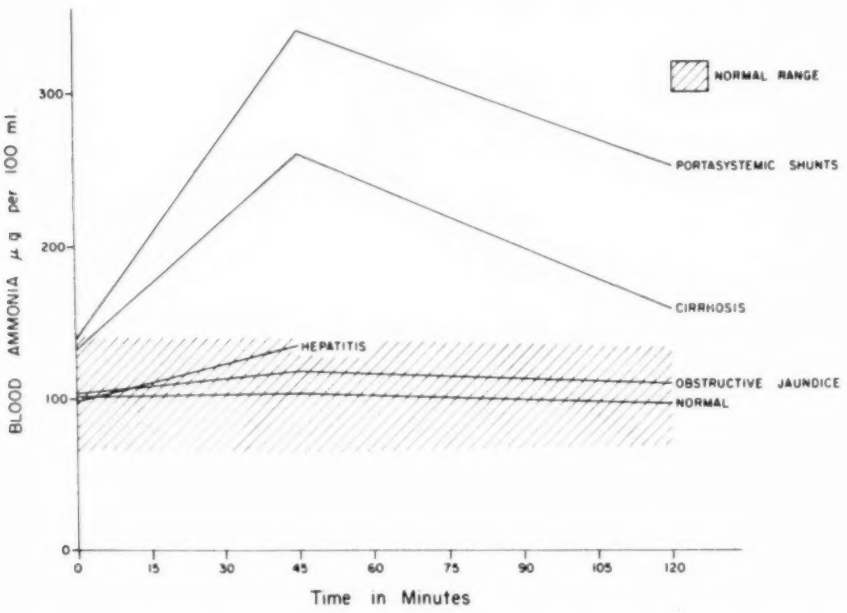

Fig. 1.—Ammonia tolerance in liver disease. The mean blood ammonia values after the oral administration of 3 Gm. of ammonium chloride are depicted for each group of patients. The hatched area represents the normal range. Mean values and standard deviations are presented in Table II.

with hepatitis and 1 of 10 with cirrhosis showed normal venous ammonia values but abnormally high arterial levels 45 minutes after ammonium chloride. Variations in the extraction of ammonia by the peripheral tissues may thus significantly influence the results of this test.

Arterial hydrogen ion concentration was not altered 45 minutes after the administration of 3 Gm. of ammonium chloride.

Nausea in varying degrees developed in most of the subjects of this study and vomiting was precipitated in 6 per cent. The results in these cases were

*The patients presented in Table III and Fig. 2 are not representative of the whole group but contain an inordinate number of patients with hepatitis with impaired ammonia tolerance and patients with cirrhosis with normal tolerance.

discarded. Mild neurologic symptoms, consisting of confusion and asterixis were precipitated transiently in 2 patients with cirrhosis.

Preliminary studies indicated that the 45 minute blood ammonia determination accurately reflected ammonia tolerance in these patients, and that the 120 minute specimen was of little additional value. Consequently this determination was omitted in approximately half of the studies reported here.

DISCUSSION

The determination of ammonia tolerance, although apparently simple, is a complicated test dependent on the simultaneous activities of several organs, and subject to modification by many factors. A brief review of ammonia metabolism will delineate some of the factors which may affect the peripheral venous ammonia concentration after the ingestion of an ammonium salt.

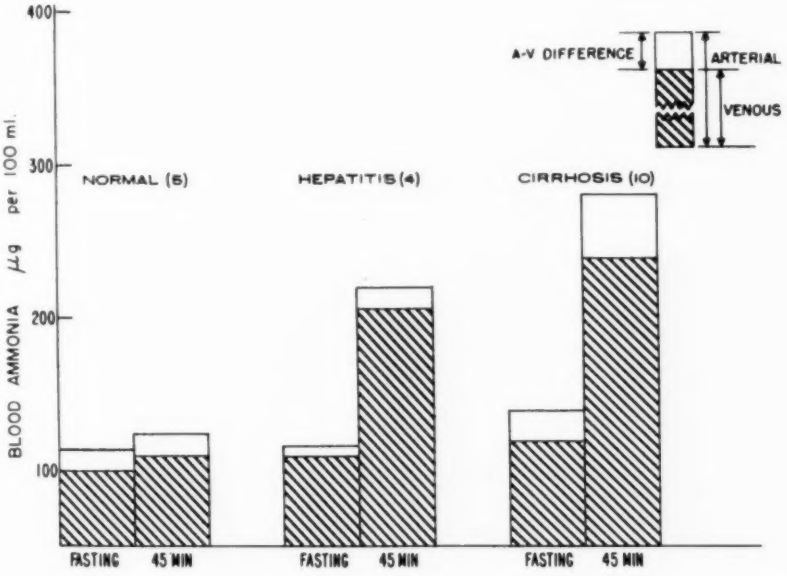

Fig. 2.—Arterial and venous ammonia tolerance tests in liver disease. The mean arterial and venous ammonia values are plotted.

Ammonia is a key intermediate in the metabolism of nitrogen. The gastrointestinal tract is the major portal of entry of ammonia which arises from the bacterial hydrolysis of urea[13] and the deamination of dietary proteins. The ammonia is carried to the liver where it is extracted[14-16] and stored as glutamine until it is utilized in the synthesis of urea or protein.[17] Besides removing ammonia from the circulation, the liver also liberates ammonia in the course of deaminating amino acids and degrading other nitrogenous compounds.[18, 19] Consequently, the ammonia content of the hepatic vein represents the sum of the unextracted portal vein ammonia and that contributed by the liver.

Although the kidney is the primary site for the excretion of nitrogen and ammonium ions from the body it also contributes to the blood ammonia level.

Renal vein ammonia levels are always higher than simultaneous arterial levels.[16, 19, 20] It appears, paradoxically, that the more ammonia formed in the kidney for excretion, the more "leaks back" into the systemic circulation.[19]

Under normal resting conditions the peripheral tissues appear to take up a small amount of ammonia. During muscular activity, however, ammonia is liberated in large quantities.[21, 22] In the face of rising arterial ammonia levels, as much as 40 per cent of the arterial ammonia may be extracted by the extremities.[23-25] Brain tissue also appears to remove ammonia from the blood, although to a lesser degree than the extremities.[23, 24, 26]

TABLE III. ARTERIAL-VENOUS AMMONIA TOLERANCE TESTS

PATIENT	FASTING BLOOD AMMONIA (μG/100 ML.) ARTERIAL	VENOUS	A-V DIFFERENCE	45 MINUTE BLOOD AMMONIA (μG/100 ML.) ARTERIAL	VENOUS	A-V DIFFERENCE
Normal.—						
1	101	98	+ 3	91	88	+ 3
2	121	109	+12	141	126	+ 15
3	128	98	+30	150	131	+ 19
4	106	94	+12	111	101	+ 10
5	108	97	+11	131	106	+ 25
Mean	113	99	+14	125	110	+ 15
Hepatitis.—						
A. R.	104	100	+ 4	181	133	+ 48
J. A.	142	131	+11	186	167	+ 19
J. F.	118	106	+12	320	308	+ 12
L. R.	105	100	+ 5	194	216	- 22
Mean	117	109	+ 8	220	206	+ 14
Cirrhosis.—						
26	141	114	+27	386	342	+ 44
27	108	90	+18	104	118	- 14
36	100	70	+30	441	231	+210
37	198	153	+45	452	408	+ 44
42	118	119	- 1	130	117	+ 13
47	132	132	0	162	164	- 2
50	96	105	- 9	130	137	- 7
58	205	169	+36	380	346	+ 34
61	147	143	+ 4	396	396	0
62	144	99	+45	225	121	+104
Mean	139	119	+20	280	238	+ 42

The result, then, of a venous ammonia tolerance test in a normal individual depends primarily on the hepatic clearance of the absorbed ammonium salt. In the cirrhotic patient, however, extra- and intrahepatic portasystemic shunting, as well as hepatic parenchymal damage, permit an elevation of the systemic blood ammonia concentration. In addition, the ability of the peripheral tissues to extract ammonia from the arterial blood varies greatly and hence may affect the results unpredictably. Finally, the antecedent diet and medications, as well as unrecognized factors, may influence the results. The venous ammonia level after the oral administration of ammonium salts, therefore, represents the net result of multiple factors. Nevertheless, our results indicate that the ammonia tolerance test correlates fairly well with certain features of liver disease.

Ammonia Tolerance in Normal Subjects.—Our control group showed no significant increase above the fasting blood ammonia concentration after the ingestion of ammonium chloride. These results are in complete accord with those of Van Coulaert[1] and Roger[3] and their co-workers, who employed larger amounts of ammonium salts (Table I). The data differ slightly from those of Kirk[2] who noted a small increase of ammonia levels 30 minutes after administration of ammonium citrate. White and co-workers,[6] who used the same dose of ammonium chloride that we employed, also noted a small increase in blood ammonia in their control subjects. These authors measured the blood ammonia

TABLE IV. SERIAL AMMONIA TOLERANCE TESTS IN VIRAL HEPATITIS

PATIENT	AGE	HEPATITIS		INTERVAL (DAYS)	SERUM BILIRUBIN (MG./100 ML.)	BLOOD AMMONIA (μG/100 ML.)		REMARKS
		SEVERITY	STAGE			FASTING	45 MIN.	
D. V.	21	Moderate	Acute		17.2	103	179	Varices on esophagoscopy
			Subsiding	7	6.0	62	64	No varices on esophagoscopy
R. K.	60	Severe	Acute		7.2	126	291	Varices on esophagoscopy
			Subsiding	8	4.0	115	193	On adrenocortical hormones
J. A.	63	Severe	Acute		20.1	131	167	?Esophageal varices by x-ray
			Subsiding	40	2.9		96	Clinically improved
J. M.	20	Moderate	Acute		14.2	122	196	Pregnant
			Subsiding	21	4.9	136	172	15 days postpartum
			Subsided	60	1.4	87	109	Clinically well
J. F.	80	Mild	Acute		5.4	106	308	No varices by esophagoscopy
			Subsiding	14	2.4	109	159	Improved clinically
			Subsided	224	0.3	115	176	Clinically well
L. R.	26	Moderate	Acute		8.8	100	216	No varices by esophagoscopy, SGOT* 1,040 units
			Subsiding	11	2.4	100	112	SGOT* 420 units

*Serum glutamic oxalacetic transaminase.

30 minutes after the test substance was administered. Interpolation of their data suggests that had the ammonia levels been measured at 45 minutes, they would have been lower than those reported. In addition, the control group included ". . . patients with chronic pulmonary or cardiac disease who had normal liver function tests." Elevated blood ammonia levels have been reported in pulmonary emphysema[27] and congestive heart failure,[28, 29] and impaired ammonia tolerance has been observed in congestive heart failure.[30]

Analysis of both arterial and venous ammonia levels in control patients (Table III) shows that the arterial level is slightly higher than the venous level in the fasting state. After ammonium chloride there is a small rise in the arterial level and no significant change in the arteriovenous difference.

These data indicate that normal subjects are able to metabolize large amounts of ammonia rapidly without evidence of toxicity or a significant increase in the systemic blood ammonia concentration. Even larger doses of ammonium chloride (4.5 to 5.1 Gm.) failed to significantly increase the venous ammonia concentration in 6 additional normal volunteers.

On the basis of these control studies we have arbitrarily defined a normal venous ammonia tolerance test as one in which the fasting level is within normal limits and in which the 45 minute level remains within the normal fasting range.

Ammonia Tolerance in Patients With Obstructive Jaundice.—Our patients with obstructive jaundice characteristically showed a normal fasting blood ammonia level and an insignificant rise after the administration of ammonium chloride. Only 1 patient exhibited impaired ammonia tolerance. This 69-year-old alcoholic, who was admitted to the hospital for recurrent jaundice 1 year after cholecystectomy, showed a high normal fasting blood ammonia followed by a small increase after administration of ammonium chloride. Except for jaundice and an enlarged liver there were no stigmata of liver disease. The serum albumin was 2.6 Gm. per 100 ml. Stricture of the common bile duct was noted at laparotomy, but liver biopsy was not done. Esophagoscopy was not performed. It is possible that this patient may have had cirrhosis of either the alcoholic or biliary type. It has not been possible to re-evaluate ammonia tolerance in this patient. Four additional patients with a clinical picture of obstructive jaundice caused by extensive metastatic involvement of the liver exhibited normal ammonia tolerance.

Previous experience with ammonia tolerance in obstructive jaundice has, with few exceptions, paralleled our own. White and co-workers,[6] however, reported upon two patients with obstructive jaundice caused by carcinoma in whom both fasting and post-ammonium chloride levels were abnormally elevated. It is interesting to note that simultaneous hepatic venous ammonia levels were within normal limits in these 2 patients. These findings suggest that the elevated systemic levels were not due to impaired hepatic extraction but to some other mechanism.

There was no correlation in our patients between the ammonia tolerance test and duration or severity of jaundice, the elevation of serum alkaline phosphatase, or the degree of Bromsulphalein retention.

It appears plain that patients with obstructive jaundice retain the capacity to clear moderate quantities of ammonia from the blood.

Ammonia Tolerance in Patients With Hepatitis.—The mean ammonia tolerance curve in hepatitis consisted of a normal fasting level followed by a small increase in blood ammonia concentration 45 minutes after the administration of ammonium chloride. Sixteen of the 22 patients displayed normal ammonia tolerance. The 6 patients with abnormal ammonia tolerance were studied serially and will be discussed briefly (Table IV).

D. V. was a 21-year-old man who developed viral hepatitis of moderate severity. Ammonia tolerance test performed on the tenth day of jaundice showed a slightly abnormal

response. Esophagoscopy at this time revealed small esophageal varices at the distal end of the esophagus. These were not visible radiographically. One week later, at a time when the jaundice had improved, a second ammonia tolerance test was normal. Subsequent esophagoscopy did not reveal esophageal varices.

R. K., a 60-year-old man, was admitted with a history suggestive of mild viral hepatitis. Liver biopsy showed severe subacute hepatic necrosis. Ammonia tolerance was grossly abnormal on the eighteenth day of jaundice. Esophageal varices were seen on esophagoscopy. After 10 days of adrenocortical steroid therapy, during which there was excellent clinical response, ammonia tolerance was improved although still abnormal. Five days later the patient died suddenly of unrelated cause. No esophageal varices were seen at autopsy.

The demonstration of esophageal varices in acute viral hepatitis confirms the observations of Palmer and Brick.[31] Other workers have found corollary evidence of increased portal venous pressure in acute viral hepatitis.[32, 33] Although the abnormality in these cases may have been related to the severity of the hepatitis, the visualization of esophageal varices suggests an alternative explanation.

J. A., a 63-year-old man, developed viral hepatitis 2 months after an exploratory thoracotomy which disclosed metastatic bronchogenic carcinoma. His course was severe, and a liver biopsy showed subacute hepatic necrosis with changes of the nature which presage the development of postnecrotic cirrhosis.[34] An ammonia tolerance test at the height of the hepatitis was minimally abnormal. Barium swallow at this time was suggestive of esophageal varices. The patient declined esophagoscopy. Six weeks later after a long course of steroid therapy and marked clinical improvement, a second test was normal. Despite subacute hepatic necrosis this patient showed only minimal ammonia intolerance. Although it is reasonable to assume that such an abnormality might be attributed to severe hepatitis the radiographic suggestion of esophageal varices again prompted another explanation.

Another patient, a pregnant woman, with hepatitis of average severity, showed moderately abnormal ammonia tolerance. Three weeks later, after premature birth of an infant, and while still overtly jaundiced, ammonia tolerance was still slightly abnormal. A third test was normal after the patient had recovered clinically.

In this patient, too, the degree of ammonia intolerance is roughly proportional to the severity of the hepatitis. The demonstration of esophageal varices in normal pregnancy[31] again suggests that portasystemic shunting may have been responsible for the impaired ammonia tolerance. Although esophagoscopy was not performed in this patient it is interesting to speculate about the possible presence of esophageal varices related to the hepatitis, the pregnancy, or both.

The other 2 patients had homologous serum jaundice which appeared to be mild clinically and histologically although one of them (L. R., Table IV) had a greatly elevated serum transaminase (SGOT). The other patient, an 80-year-old man with a hiatus hernia, had grossly abnormal ammonia tolerance. It was still slightly abnormal 7 months later despite good health and completely normal liver function (J. F., Table IV). Although esophageal varices were not observed during esophagoscopy in these 2 patients, it has been emphasized that the inability to visualize varices esophagoscopically does not exclude their presence, since they have been reported to come and go without apparent cause.[35]

Simultaneous arterial and venous ammonia tolerance tests in 4 patients with hepatitis showed that in 3 patients both the arterial and venous ammonia levels rose about equally (Table III). In the fourth patient an increased arteriovenous uptake resulted in a normal 45 minute venous ammonia level despite an increased arterial level. This phenomenon may have occurred in some of the 15 patients with hepatitis who had normal ammonia tolerance by standards based on venous values.

Previous workers have found normal ammonia tolerance in patients with hepatitis except for White and co-authors,[6] who reported both elevated ammonia levels and abnormal ammonia tolerance. Furthermore, they demonstrated that ammonia concentration was usually greater in the hepatic vein than in peripheral venous blood both before and after ammonium chloride. Although they studied only 3 patients in this manner, their findings suggested that the rise in peripheral blood ammonia may have been due to decreased extraction of ammonia by the liver. In one of their patients (P. A.), however, peripheral venous ammonia levels appeared to rise without a concomitant rise in the hepatic venous ammonia concentration. This suggests that a mechanism other than hepatocellular dysfunction was responsible for the abnormal ammonia tolerance.

Our studies confirm their observations that abnormal ammonia tolerance may occur in viral hepatitis. There is little doubt that impaired ammonia tolerance may be caused by severe parenchymal damage in hepatitis. Our findings indicate, however, that esophageal varices do exist in acute viral hepatitis and that such portasystemic shunts may offer another explanation for increased systemic blood ammonia concentrations. Furthermore, it is important to note that normal ammonia tolerance may exist despite severe hepatocellular impairment as measured by the usual tests of liver function.

Ammonia Tolerance in Hepatic Fibrosis and/or Fatty Infiltration.—The normal pattern obtained in the patients with portal fibrosis and/or fatty infiltration of the liver confirm the findings of Stahl and collaborators.[4] Each of these patients had hepatomegaly, and a few had spider angiomata or splenomegaly. Although Bromsulphalein retention was elevated in almost all of them, and increased serum bilirubin levels were present in half, none exhibited evidence of severe hepatic insufficiency or portasystemic collateral circulation. Esophageal varices were not visualized in the 4 patients examined by esophagoscopy.

Ammonia Tolerance in Cirrhosis.—Abnormal ammonia tolerance tests were observed in 50 of the 60 cirrhotic patients. These tests were characterized by a fasting venous ammonia level in the high normal range, a large rise at 45 minutes, and a partial return to normal at 2 hours. Although the mean fasting blood ammonia level for the whole group fell within the normal range, it was elevated in more than one third of the individual patients.

The simultaneous measurement of arterial and venous ammonia concentrations after administration of ammonium chloride disclosed that usually the arterial ammonia levels were enormously raised and that, despite large and

variable arteriovenous ammonia differences, the peripheral venous levels were abnormally elevated. Occasionally, however, a large ammonia uptake by the peripheral tissues resulted in normal 45 minute venous ammonia values which could be misinterpreted as normal ammonia tolerance. The factors which determine the magnitude of the arteriovenous difference are not apparent.

Analysis of liver function revealed that there was no significant correlation between the ammonia tolerance test and the serum bilirubin, Bromsulphalein retention, thymol turbidity, cephalin flocculation, or alkaline phosphatase determinations. This poor correlation suggested that the ammonia tolerance test is not a sensitive measure of hepatic parenchymal function per se. There was, however, a significant correlation between the degree of ammonia tolerance and the serum albumin concentration (coefficient of correlation minus 0.386;

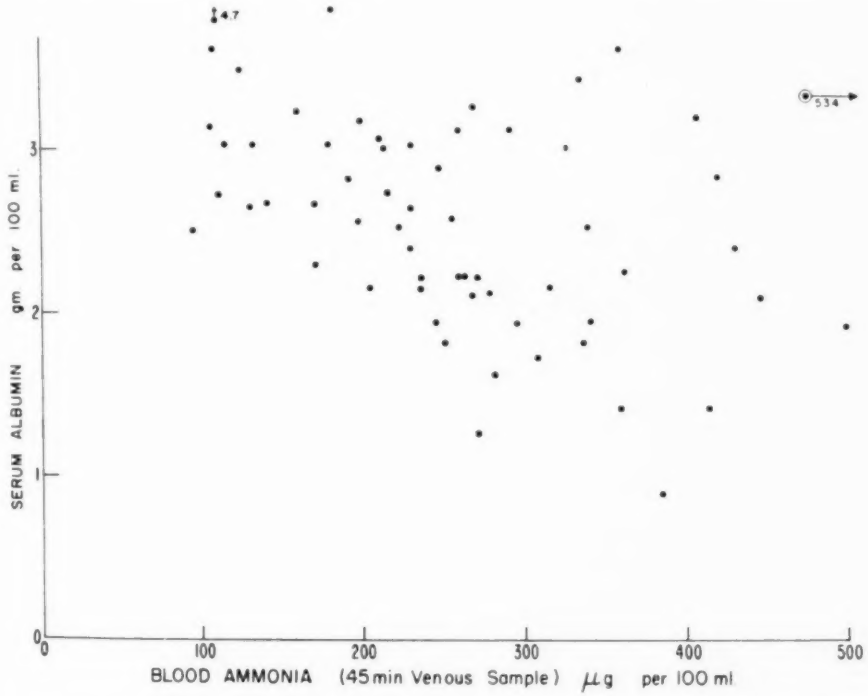

Fig. 3.—Serum albumin concentration and ammonia tolerance in cirrhosis. The coefficient of correlation is −0.386; $p < 0.01$.

< 0.01). (Fig. 3.) There was no such relationship with the total serum protein or globulin levels. This correlation did not hold for the other types of liver disease studied. Although the serum albumin concentration is generally regarded as a test of liver function, it appears to serve also as a rough index of the general state of health and nutrition of the patient. The degree of hypoalbuminemia may thus reflect the severity or chronicity of the cirrhotic process and conceivably may parallel the degree of development of the portal collateral circulation. There is no evidence in the literature to suggest that blood ammonia concentrations are directly related to serum albumin levels.

The degree of ammonia intolerance also correlated with certain physical findings which are characteristic of cirrhosis (Fig. 4). A highly significant correlation was observed between abnormal ammonia tolerance and the presence of visible abdominal collateral veins ($p < 0.001$). Indeed, the 4 patients with the Cruveilhier-Baumgarten syndrome exhibited some of the most abnormal tests in the whole group. Less significant correlations were observed with splenomegaly ($p < 0.01$), ascites ($p < 0.05$), and spider angiomata ($p < 0.05$). Each of these physical findings is usually associated with portal hypertension.

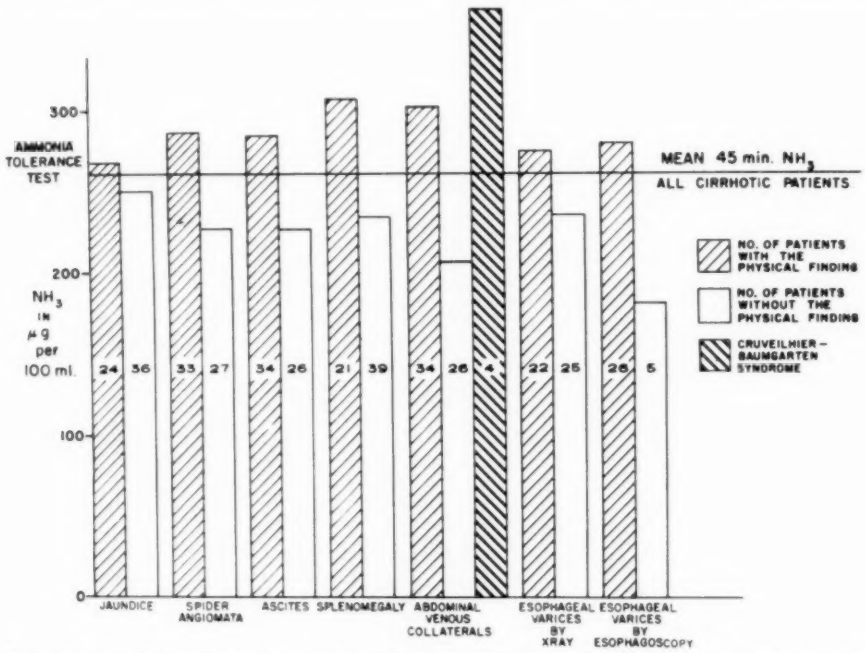

Fig. 4.—Correlation of certain clinical findings and ammonia tolerance in cirrhosis. The height of the bars represents the mean 45 minute blood ammonia concentration. The number indicates the number of patients in each group.

It has been noted by some that esophageal varices are more frequently associated with spider angiomata than with any other physical sign.[38] Our data did not confirm this finding.

There was no significant relationship between abnormal ammonia tolerance and the presence of esophageal varices as demonstrated by radiography or esophagoscopy. Although ammonia tolerance was much more abnormal in the patients with esophagoscopically proved varices (Fig. 4), these data are not statistically significant in view of the small number of patients without esophageal varices.

There was no correlation between ammonia tolerance and the degree of hepatomegaly, the severity of jaundice or the presence of edema in these patients. These findings suggest that abnormal ammonia tolerance is not caused by hepatic parenchymal dysfunction, per se, but, that it is related to the degree of development of portal hypertension and collateral circulation as reflected by such findings as abdominal collateral veins, splenomegaly, and spider angiomata.

The 10 cirrhotic patients with normal ammonia tolerance fall into 2 well-defined groups. The first consisted of 6 patients in whom the diagnosis of cirrhosis was not evident clinically, but in whom liver biopsy showed a mild cirrhosis without evidence of degeneration or inflammation. None of the 6 were jaundiced. Only 1 exhibited spider angiomata. All had hepatomegaly but none had splenomegaly, ascites, or abdominal collateral veins. One was thought to have esophageal varices by radiologic examination and 1 by esophagoscopy. Serum bilirubin levels were normal in 5 and minimally elevated in the sixth. Bromsulphalein retention was normal in 4, borderline in 1, and severely abnormal only in 1 patient with cardiac decompensation. Serum albumin was above 3 Gm. per 100 ml. in 4 of them. Ammonia tolerance tests repeated 1 year later in 4 of these patients remained normal in 3, and was minimally abnormal in the fourth (Table III, Patients 27, 42, 47, and 50). This group of patients appears similar to that reported by White and collaborators[6] in whom cirrhosis without demonstrable collateral channels was present and in whom normal ammonia tolerance was found.

The second group consisted of 4 alcoholic patients with frank clinical and laboratory evidence of cirrhosis. Liver function studies were grossly abnormal. Esophageal varices were demonstrated in the 3 patients examined by esophagoscopy. Liver biopsies in each of these patients showed advanced active Laennec's cirrhosis. Although each of these 4 patients had normal ammonia tolerance on the basis of a normal 45 minute venous ammonia level, 3 of them showed a rise of more than 35 μg per 100 ml. above the fasting level. The most probable explanation for these normal tests is illustrated by one of our patients (Table III, Patient 62) in whom a subsequent arterial-venous ammonia tolerance test showed abnormal ammonia tolerance by arterial standards but normal tolerance based on venous ammonia levels. Stahl and co-workers[39] have recently established that false normal ammonia tolerance may result from enhanced extraction of ammonia by peripheral tissues as evidenced by an increased arteriovenous difference. These studies stress the greater validity of arterial ammonia levels in the performance of ammonia tolerance tests.

Ammonia Tolerance in Portasystemic Anastomoses.—Grossly abnormal ammonia tolerance tests occurred in all 18 patients with cirrhosis with portacaval or splenorenal anastomoses. The 45 minute blood ammonia level in 15 far surpassed the mean for the cirrhotic patients. These 2 groups were similar by clinical and laboratory criteria, although the mean Bromsulphalein retention was less abnormal in the "shunt" group than in the cirrhotic patients. These findings emphasize the role of shunting in the ammonia tolerance test. In addition they confirm the report of Eiseman and associates[40] that ammonia intolerance increases after portasystemic shunts are surgically constructed.

One of the major points of controversy about ammonia tolerance has been the question of whether decreased ammonia tolerance is due to impaired hepatocellular function or to portasystemic shunting.[1, 2, 4, 6] What is the relative importance of these 2 factors?

It is well established that patients with portasystemic anastomoses, but without apparent liver damage, may exhibit impaired ammonia tolerance[6, 41, 42]

and may sometimes show the clinical picture of portal encephalopathy. These situations have been duplicated in experimental animals.[43-45]

On the other hand, there is no question that the capacity of the liver to perform its normal functions, including regulation of ammonia levels, may be lost by complete hepatic destruction such as might occur in overwhelming hepatic necrosis in humans, or in total hepatectomy in animals.[46] It follows that there exists a degree of hepatic damage which destroys only the reserve capacity of ammonia extraction but which permits "normal" extraction until faced with a sudden large ammonia load as occurs in an ammonia tolerance test. The amount of damage required to exhaust this reserve capacity is unknown. Hepatic perfusion techniques have shown that ammonia extraction remains normal despite severe parenchymal necrosis.[47] On the basis of experiments in dogs, which are probably not directly applicable, in excess of 75 to 80 per cent of the liver may be removed without impairing ammonia tolerance or urea synthesis.[14, 48] In man, most investigators have shown that ammonia tolerance is not impaired in viral hepatitis. Furthermore, data have been presented to show that in severe cirrhosis the liver retains its capacity to extract large amounts of ammonia from the portal blood[2, 5, 6] and even in the presence of abnormal ammonia tolerance these patients may synthesize urea normally.[2, 6]

The maximal dose of oral ammonium salts which can be tolerated in the normal human without elevating venous ammonia concentrations is unknown. We have noted no significant rise after 3 to 5 Gm. of ammonium chloride and Zimmerman[49] has observed similar results with 6 Gm. Normally the portal venous ammonia concentration in humans is 2 to 4 times the peripheral levels[19] and may rise to tremendous levels after oral administration of ammonium salts.[2] It must be assumed that the portal vein ammonia levels may sometimes exceed the maximal hepatic clearing capacity, even in normal subjects. If one assumes that the liver extracts a constant percentage of the ammonia presented to it, as appears to be true in dogs,[15, 50] then hepatic venous ammonia levels may rise to abnormal heights in spite of continued "normal" clearance. If, in the presence of hepatitis or cirrhosis, a dose of ammonium chloride were employed which approached the maximal capacity in normal subjects, then increased amounts of ammonia would escape extraction by the liver. Our studies suggest that the dose of ammonium chloride which we employ allows a sizable reserve margin in normal subjects. This tends to minimize the effect of parenchymal damage and, consequently, to exaggerate the shunting factor in patients with hepatic disorders.

Our studies indirectly confirm earlier reports that ammonia tolerance serves primarily as a guide to the amount of collateral flow between the portal and the systemic circulation. Although some impairment of ammonia tolerance may occur in patients with severe hepatic parenchymal damage, the volume of portal collateral shunting appears to be a much more important factor. The test, as employed here, is quite simple and safe, and when used as a screening test can be reduced to a single blood ammonia determination after the administration of the ammonium salt. The use of arterial ammonia levels enhances the accuracy

of this determination. This test, or modifications of it, may prove to be of clinical value as a "medical" means of estimating the portal collateral flow. Further studies are required to evaluate more fully the specificity and sensitivity of this test and to delineate further ammonia clearance in the liver and in the peripheral tissues.

SUMMARY

Normal ammonia tolerance was observed in patients with obstructive jaundice, fibrosis and/or fatty infiltration of the liver and in most patients with acute hepatitis. In several of the patients with hepatitis in whom abnormal ammonia tolerance was found, esophageal varices, which may have accounted for the impaired ammonia tolerance, were demonstrated. Ammonia tolerance was abnormal in 83 per cent of 60 patients with cirrhosis. Impaired ammonia tolerance in cirrhosis appeared to be related more closely to the degree of development of portal collateral circulation than to hepatic functional damage. In patients with portacaval shunts ammonia tolerance was much more abnormal than in the cirrhotic group, despite less severely deranged liver function.

Abnormal ammonia tolerance appears to be primarily a gross index of the amount of portasystemic anastomoses. Severe hepatic parenchymal damage may also impair ammonia clearance, although to a lesser degree.

Simultaneous measurements of arterial and venous ammonia levels disclosed that although ammonia tolerance tests performed on venous blood are usually valid, increased arteriovenous uptake of ammonia may occasionally result in false negative tests. Arterial ammonia levels are consequently of greater diagnostic value.

The ammonia tolerance test is a simple, safe, and reliable screening test for the estimation of the presence of portasystemic collateral circulation.

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REFERENCES

1. Van Caulaert, C., and Deviller, C.: Ammoniémie expérimentale après ingestion de chlorure d'ammonium chez l'homme à l'état normal et pathologique, Compt. rend. Soc. de biol. **111:** 50, 1932.
2. Kirk, E.: Amino Acid and Ammonia Metabolism in Liver Diseases, (supp.) Acta med. scandinav. **77:** 1, 1936.
3. Roger, S., and Stahl, J.: Corrélation entre l'épreuve d'ammoniémie provoquée et l'histopathologie hépatique, Comp. rend. Soc. de biol. **146:** 1786, 1952.
4. Stahl, J., Roger, S., and Witz, J.: Essai d'interprétation du méchanisme de l'épreuve d'hyperammoniémie provoquée chez les cirrhotiques, Compt. rend. Soc. de biol. **146:** 1787, 1952.
5. Stahl, J., Roger, S., and Bockel, R.: Signification de l'ammoniémie provoquée au cours de diverse affections hépatiques, Acta gastro-enterol. belg. **18:** 846, 1955.
6. White, L. P., Phear, E. A., Summerskill, W. H. J., and Sherlock, S.: Ammonium Tolerance in Liver Disease—Observations Based on Catheterization of the Hepatic Veins, J. Clin. Invest. **34:** 158, 1955.
7. Seligson, D., and Hirahara, K.: The Measurement of Ammonia in Whole Blood Erythrocytes and Plasma, J. LAB. & CLIN. MED. **49:** 962, 1957.
8. Gaebler, O. H.: Determination of Bromsulphalein in Normal, Turbid, Hemolyzed and Icteric Serums, Am. J. Clin. Path. **15:** 452, 1945.

9. Klatskin, G., and Yesner, R.: Factors in the Treatment of Laennec's Cirrhosis. I. Clinical and Histological Changes Observed During a Control Period of Bed Rest, Alcohol Withdrawal, and a Minimal Basic Diet, J. Clin. Invest. **28:** 723, 1949.
10. Gornall, A. G., Bardawill, C. J., and David, N. M.: Determination of Serum Proteins by Means of the Biuret Reaction, J. Biol. Chem. **177:** 751, 1949.
11. Cohn, C., and Wolfson, W. Q.: Studies in Serum Proteins. II. A Rapid Clinical Method for the Rapid Determination of Albumin or Globulin in Serum or Plasma, J. LAB. & CLIN. MED. **33:** 367, 1948.
12. Reitman, S., and Frankel, S.: A Colorimetric Method of Serum Glutamic Oxalacetic and Glutamic Pyruvic Transaminases, Am. J. Clin. Path. **28:** 56, 1957.
13. Dintzis, R. Z., and Hastings, A. B.: The Effect of Antibiotics on Urea Breakdown in Mice, Proc. Nat. Acad. Sc. **39:** 571, 1953.
14. Islami, A. H., Pack, G. T., Miller, R. R., Vanamee, P., Randall, H. T., and Roberts, K. E.: Postoperative Course Following Total Right Hepatic Lobectomy, Surgery **39:** 551, 1956.
15. Artz, C. P., Stanley, T. V., Jr., Eure, W. R., Langford, H. G., and Snavely, J. R.: Inflow and Outflow Changes in Ammonia Concentrations of Liver, Muscle and Brain, Surgery **44:** 22, 1958.
16. Nelson, R. M., and Seligson, D.: Studies on Blood Ammonia in Normal and Shock States, Surgery **34:** 1, 1953.
17. Duda, G. D., and Handler, P.: Kinetics of Ammonia Metabolism in Vivo, J. Biol. Chem. **232:** 303, 1956.
18. Fahey, J. L., Nathans, D., and Rairigh, D.: Effect of L-Arginine on Elevated Blood Ammonia Levels in Man, Am. J. Med. **23:** 860, 1957.
19. Bessman, S. P.: Ammonia Metabolism in Animals, *in* McElroy, W. D., and Glass, B., editors: A Symposium on Inorganic Nitrogen Metabolism, Baltimore, 1956, Johns Hopkins Press, p. 408.
20. McDermott, W. V., Jr., Adams, R. D., and Riddell, A. G.: Ammonia Metabolism in Man, Ann. Surg. **140:** 539, 1956.
21. Parnas, J. K., Mozolowski, W., and Lewinski, W.: Ueber den Ammoniakgehalt und die Ammoniaksbildung im Blute. IX. Der Zusammenhang des Blutammoniaks mit der Muskelarbeit, Biochem. Ztschr. **188:** 15, 1927.
22. Schwartz, A. D., Lawrence, W., Jr., and Roberts, K. E.: Elevation of Peripheral Blood Ammonia Following Muscular Exercise, Proc. Soc. Exper. Biol. & Med. **98:** 548, 1958.
23. Bessman, S. P., and Bradley, J. E.: Uptake of Ammonia by Muscle. Its Implications in Ammoniagenic Coma, New England J. Med. **253:** 1143, 1955.
24. Webster, L. T., Jr., and Gabuzda, G. J.: Ammonium Uptake by the Extremities and Brain in Hepatic Coma, J. Clin. Invest. **37:** 414, 1958.
25. Tyor, M. P., and Wilson, W. P.: Peripheral Biochemical Changes Associated With the Intravenous Administration of Ammonium Salts in Normal Subjects, J. LAB. & CLIN. MED. **51:** 592, 1958.
26. Eiseman, B., and Clark, G. M.: Studies in Ammonia Metabolism. III. The Experimental Production of Coma by Carotid Arterial Infusion of Ammonium Salts, Surgery **43:** 476, 1958.
27. Dutton, R., Jr., Nicholas, W., Fisher, C. J., and Renzetti, A. D., Jr.: Blood Ammonia in Pulmonary Emphysema, Clin. Res. Proc. **6:** 316, 1958.
28. Bessman, A. N., and Evans, J. M.: The Blood Ammonia in Congestive Heart Failure, Am. Heart J. **50:** 715, 1955.
29. Calkins, W. G., and Delp, M.: A Study of Blood Ammonia Concentrations in Congestive Heart Failure With Report of Three Cases, J. Kansas M. Soc. **57:** 538, 1956.
30. Conn, H. O.: Ammonia Tolerance in Congestive Heart Failure. To be published.
31. Palmer, E. D., and Brick, I. B.: Esophageal Varices: Causes Other Than Cirrhosis and Portal Vein Block, J. Am. Geriat. Soc. **3:** 681, 1955.
32. Reichman, S., and Davis, W. D., Jr.: The Splenic Approach to the Portal Circulation. Intrasplenic and Intrahepatic Tissue Pressure Measurements in Acute and Convalescent Hepatitis, Gastroenterology **33:** 609, 1957.
33. Krook, H.: Circulatory Studies in Liver Disease, (Supp.) Acta med. scandinav. **156:** 1, 1956.
34. Klatskin, G.: Subacute Hepatic Necrosis and Postnecrotic Cirrhosis Due to Anicteric Infections With the Hepatitis Virus, Am. J. Med. **25:** 333, 1958.
35. Palmer, E. P.: On the Natural History of Esophageal Varices, Ann. Int. Med. **47:** 18, 1957.
36. Moscowitz, E.: Laennec's Cirrhosis. Its Histiogenesis With Special Reference to the Role of Angiogenesis, Arch. Path. **45:** 187, 1948.
37. Popper, H., Elias, H., and Petty, D. E.: Vascular Pattern of the Cirrhotic Liver, Am. J. Clin. Path. **22:** 717, 1952.

38. Brick, I. B., and Palmer, E. D.: Esophageal Varices and Vascular Spiders (Nevi araneosi) in Cirrhosis of the Liver, J. A. M. A. **155**: 8, 1954.
39. Stahl, J., Bockel, R., Imler, M., Kurtz, D., and Rohmer, F.: Étude conjointe de l'ammoniémie artérielle et veineuse dans la cirrhose. I. La signification de l'ammoniémie artérielle et veineuse au cours de l'épreuve d'ammonémie provoquée par ingestion de sels ammoniacaux. Deductions physiopathologiques et cliniques, Rev. franc., Études clin. et biol. **4**: 447, 1959.
40. Eiseman, F., Lindeman, G. M., and Clark, G. M.: Clinical Evaluation of the Ammonium Citrate Tolerance Test for Determining the Patency of a Portacaval Shunt, J. Lab. & Clin. Med. **48**: 579, 1956.
41. Fuld, H.: Über die Diagnostische verwertbarkeit von Ammoniakbestimmungen im Blut, Klin. Wchnschr. **12**: 1364, 1933.
42. McDermott, W. V., Jr., and Adams, R. D.: Episodic Stupor Associated With an Eck Fistula in the Human With Particular Reference to the Metabolism of Ammonia, J. Clin. Invest. **33**: 1, 1954.
43. Monguio, J., and Krause, F.: Ueber die Bedeutung des NH_3-gerhaltes des Blutes fur die Beurteilung der Leberfunktion, Klin. Wchnschr. **13**: 1142, 1934.
44. Mann, J. D., Bollman, J. L., Huizenga, K. A., Ferrar, T., and Brindley, J. H.: Blood Ammonia; Experimental and Clinical Study in Abnormalities of the Liver and Portal Circulation, Gastroenterology **27**: 399, 1954.
45. Levine, R. S., and Rigler, S. P.: Studies on the Ammonia Tolerance Curve in Dogs With Portacaval Shunts, Surg. Forum **8**: 235, 1957.
46. Bollman, J. L., and Mann, F. C.: Studies on the Physiology of the Liver. XVIII. The Effect of Removal of the Liver on the Formation of Ammonia, Am. J. Physiol. **92**: 92, 1930.
47. Fiske, C. H., and Karsner, H. T.: The Effect of Acute Destructive Lesions of the Liver on Its Efficiency in the Reduction of the Ammonia Content of Blood, J. Biol. Chem. **18**: 381, 1914.
48. Mann, F. C.: The Effects of Complete and of Partial Removal of the Liver, Medicine **6**: 419, 1927.
49. Zimmerman, H. J., Korn, R. J., and Weinstein, H. G.: Observations on the Source of Elevated Plasma Ammonia Levels in Hepatic Insufficiency, Am. J. M. Sc. **231**: 177, 1956.
50. Wise, H. M., Jr., Knecht, A. T., Jr., Pence, D., and Ondash, B. S.: Observations on the Function of the Liver in Experimental Traumatic Shock, Surgery **45**: 274, 1956.

THE SODIUM EXCRETION DEFECT IN HEPATIC CIRRHOSIS AS STUDIED BY THE USE OF A STEROIDAL LACTONE

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THE failure of patients with hepatic cirrhosis and ascites to excrete sodium normally has been repeatedly demonstrated.[1-3] Sodium retention in such patients, furthermore, occurs regardless of whether the administered load is given orally or intravenously, as isotonic or hypertonic solution.[1, 4] Renal function studies indicate that such sodium retention is most frequently due to avid tubular reabsorption rather than to decreased glomerular filtration.[5] High urinary excretion of aldosterone has been found to be associated with ascites and edema formation in such individuals.[6, 7]

Recently a group of steroidal lactone compounds, 3-(3-oxo-17, β-hydroxy-4-androsten-17-a-yl) proprionic acid lactone (SC5233) and its 19 nor-analogue (SC8109) have been reported to block the effects of aldosterone upon urinary sodium and potassium excretion in both animals and man.[8, 9]

If the failure of the patient with cirrhosis and ascites to excrete an acute saline load is due to excessive circulating aldosterone and if the steroidal lactone compounds are competitive inhibitors of aldosterone, the administration of these compounds to cirrhotic patients with ascites should revert their excretion of sodium in response to hypertonic saline to a more normal pattern. The investigation of this hypothesis is presented in this report.

MATERIALS AND METHODS

Five studies in 4 patients with cirrhosis of the liver and ascites were carried out. All were given a diet which contained approximately 9 mEq. of sodium, and daily 24 hour urine specimens were collected. After 2 or more days on this regimen, 2.5 c.c. of 5 per cent saline per kilogram of estimated dry body weight was administered by venoclysis over a 1 hour period. All patients had fasted for 9 hours and received no water during the infusion and for 3 hours thereafter. Fluid intake was maintained constant at 2,000 ml. per day for patients E. F. and B. C. but was taken ad libitum by the others. Urine collections and serum samples were taken before and after the infusion, urine being collected for 1 hour before the infusion and at 1, 4, and 23 hours after the infusion was begun. Beginning on the day after the initial infusion 250 mg. of SC8109† was administered orally every 6 hours for 4 days. On the fourth day the infusion was repeated and similar samples were again collected.

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Urine collections were then continued for 3 days after SC8109 was withdrawn. One patient received SC8109 orally in the usual dose and, in addition, was given 250 mg. per day in sesame oil intramuscularly.

The sodium and potassium analyses were done by flame photometry using an internal standard.

RESULTS

The results of this study have been grouped into 2 categories: the acute responses to the hypertonic saline load, and the more chronic, daily changes caused by SC8109 administration. The compilation of the data is shown in Table I.

Acute Responses.—Infusion of hypertonic saline in these cirrhotic patients during the control periods revealed the expected poor natriuretic response (Table I). Three patients (4 studies) excreted 7.3 per cent or less of the infused sodium during the succeeding 24 hour period and 1 patient excreted 17 per cent of the load. The urine potassium changes for the 24 hour period after infusion are considered less reliable because the dietary intake was not controlled. The results obtained during the 4 hour fasting period after the infusion provide valid data, however, and potassium excretion during this period was varied. The amount excreted per hour fell after infusion in 3 studies, was unchanged in 1 and rose in 1 study. Potassium concentration was likewise variable but sharp increases were noted during the 4 hours after infusion in 3 studies while no change and a decrease in concentration occurred, respectively, in each of the others.

The administration of SC8109 resulted in a marked increase in sodium excretion in all patients both during and after the infusion (Table I, Fig. 1). This was accomplished by an increase in urine volume at the 1 and 4 hour collections in all except 1 period. The use of a larger dose of SC8109 in 1 patient (B. C.) resulted in no increase in net sodium excretion but decreased potassium excretion occurred which accounted for a sharp increase in Na:K ratio. The changes in potassium were neither as consistent nor as marked as the sodium changes. In 4 of the 5 studies the administration of SC8109 resulted in an increase in total potassium excretion during the 4 hours after the hypertonic saline infusion as compared to the previous experiments. In the fourth patient there was a decrease in urinary potassium. Despite this the concentration of urinary potassium was generally lower during and immediately after the second infusion in all patients. Although some of the decrease in concentration could be accounted for by increases in urine volume, the change in concentration exceeded the volume change in the 4 studies in which the decrease was exhibited. Urinary excretion of potassium was less in patient B. C. when he was receiving the larger dose of SC8109.

The serum potassium concentrations showed no typical pattern of change but were generally higher during the infusion after drug administration (Table I). Conversely, the serum sodium was generally lower during this latter infusion. The rise in serum sodium following the hypertonic saline was not altered by the drug.

TABLE I.

TIME		URINE						SERUM		
DAYS	HOURS	Na mEq./L.	K mEq./L.	Vol. c.c.	Total Na mEq.	Total K mEq.	Na:K	Na mEq./L.	K mEq./L.	U:P Na
E. F.										
1										
2										
3		17.4	18.8	3,000	52.2	56.4	0.93			
4	0	37.6	65.5	45	1.7	2.9	0.58	137	3.7	0.28
	1	78.6	75.2	62	4.9	4.6	1.06	138	3.8	0.57
	2							141	4.0	
	4	163.0	58.5	195	31.7	11.4	2.78	142	4.2	1.15
	23	63.3	37.0	695	43.9	25.7	1.71			
	Total			997	82.8	44.6	1.86			
5*		41.5	10.5	2,900	120.0	30.4	3.94			
6*		63.9	10.7	2,265	144.0	24.2	5.95			
7*		64.7	8.9	2,400	155.0	21.4	7.24	133	4.1	
8*	0	169.0	19.5	92	15.5	1.8	8.65	133	4.3	1.27
	1	211.0	23.1	100	21.1	2.3	9.13	137	4.6	1.52
	2							138	4.5	
	4	243.0	28.7	270	65.6	7.7	8.47	133	4.0	1.83
	23	74.0	11.1	1,800	133.0	19.9	6.68			
	Total			2,262	235.2	31.7	7.41			
9		55.5	10.9	3,290	183.0	35.9	5.09			
10		25.6	15.9	3,160	80.9	50.2	1.61			
11		14.3	19.7	2,900	41.5	57.1	0.73			
B. C. (1)										
1										
2		2.2	24.1	1,420	3.1	34.2	0.09			
3		0.8	20.8	1,930	1.4	40.1	0.03	137	3.9	
4	0	1.0	37.8	93	0.1	3.5	0.03	138	4.6	0.007
	1	0	0	0	0	0	—	140	4.3	—
	2							141	4.2	
	4	5.0	134.5	103	0.5	13.9	0.04	141	4.2	0.04
	23	3.9	54.5	660	2.6	36.0	0.07			
	Total			856	3.2	53.4	0.06			
5*		33.2	39.0	1,940	64.4	75.7	0.85			
6*		43.8	35.3	1,920	84.1	67.8	1.24			
7*		46.3	29.4	1,950	90.3	57.3	1.58			
8*	0	74.1	102.5	7	0.5	0.7	0.72	133	4.5	0.56
	1	85.4	105.8	92	7.9	9.7	0.81	138	4.8	0.62
	2							139	4.2	
	4	120.0	102.0	155	18.6	15.8	1.18	129	4.6	0.93
	23	91.9	44.0	1,040	95.6	45.8	2.09			
	Total			1,290	122.5	72.0	1.70			
9		21.0	40.0	1,620	34.0	64.8	0.52	132	3.9	
10		0.8	44.3	1,440	1.2	63.8	0.02			
11										
B. C. (2)										
1		3.6	37.0	1,270	4.5	47.0	0.10			
2		0.7	22.6	1,320	0.9	29.8	0.03			
3		2.1	41.5	1,400	2.9	58.1	0.05			
4	0	1.8	68.3	55	0.1	3.8	0.03	137	4.2	0.013
	1	0.5	49.0	31	0.02	1.5	0.01	140	3.9	0.003
	2							142	4.0	
	4	3.4	122.0	39	0.1	4.8	0.02	137	4.1	0.025
	23	9.2	49.3	760	7.0	37.5	0.19			
	Total			885	7.22	47.6	0.15			
5†		55.6	17.6	1,870	104.0	32.9	1.69			
6†		57.0	24.4	2,050	117.0	50.0	1.14			
7†		63.1	32.3	2,280	144.0	73.6	0.85			

*SC8109.
†SC8109 orally and intramuscularly.
‡Incomplete.
§Hemolyzed.

TABLE I—CONT'D

TIME		URINE						SERUM		
DAYS	HOURS	Na mEq./L.	K mEq./L.	Vol. c.c.	Total Na mEq.	Total K mEq.	Na:K	Na mEq./L.	K mEq./L.	U:P Na
8†	0	74.8	31.7	121	9.1	3.8	2.39	135	4.0	0.553
	1	126.0	43.8	52	6.6	2.3	2.87	139	5.1	0.906
	2							142	4.9	
	4	158.0	41.3	133	21.0	5.5	3.92	139	5.0	1.135
	23	108.0	24.8	1,250	135.0	31.0	4.36			
	Total			1,556	171.7	42.6	4.03			
9		34.8	62.3	1,560	54.2	97.2	0.56			
10		2.2	28.8	1,780	3.9	51.3	0.08			
11		0.7	43.3	1,440	1.0	62.4	0.02			
A. Z.										
1										
2		0	43.9	930	0	40.8	0			
3		0	44.1	1,010	0	44.5	0	135	4.3	
4	0	0	47.9	73	0	4.0	0	134	4.0	0
	1	1.6	70.3	41	0.07	2.9	0.02	139	4.3	0.012
	2							138	4.2	
	4	4.2	81.9	97	0.4	7.9	0.05	139	4.2	0.030
	23	14.6	68.5	750	10.9	51.4	0.21			
	Total			961	11.4	66.2	0.17			
5*		52.0	50.0	1,100	57.2	55.0	1.04			
6*		63.1	33.1	1,340	84.6	44.4	1.91			
7*		64.3	29.7	1,810	116.4	53.8	2.16			
8*	0	113.0	41.9	59	6.7	2.5	2.68	132	4.3	0.86
	1	146.0	39.3	86	12.6	3.4	3.71	134	4.3	1.09
	2							134	4.6	
	4	174.0	49.4	230	40.0	11.4	3.51	135	4.7	1.29
	23	110.0	54.8	1,070	118.0	58.6	2.02			
	Total			1,445	177.3	75.9	2.33			
9		68.7	52.3	810‡	56.6	42.4	1.31			
10		35.1	73.0	880	30.9	64.2	0.48			
11		24.4	58.0	1,000	24.4	58.0	0.42			
L. M.										
1		32.8	37.4	1,724	56.5	64.5	0.88			
2		17.6	16.5	840	14.8	13.9	1.06			
3		6.1	8.9	700	4.3	6.2	0.69			
4	0	34.8	26.5	102	3.5	2.7	1.30	133		0.262
	1	0.3	0.2	7	0.00	0.00	—	133		0.002
	2							131		
	4	0.1	1.3	68	0.07	0.09	—	132		0.0008
	23	15.0	5.2	795	11.9	4.1	2.90			
	Total			972	15.5	6.9	2.24			
5*		68.9	13.7	1,860	128.2	25.5	5.05			
6*		41.0	9.0	1,415	57.6	12.7	4.54			
7*		23.1	8.1	1,075	24.8	8.7	2.85			
8*	0	5.5	2.6	80	0.44	0.2	2.20	133	3.3	0.041
	1	2.1	1.8	34	0.07	0.06	—	132	§	0.016
	2							133	3.2	
	4	3.4	4.0	64	0.22	0.26	0.85	132	3.3	0.026
	23	34.0	8.6	1,660	56.4	14.3	3.94			
	Total			1,838	57.1	14.8	3.86			
9		21.8	9.2	1,275	27.8	11.7	2.38			
10		16.9	12.1	1,360	23.0	16.5	1.39			
11		7.2	21.1	1,780	12.8	36.6	0.35			

The ratio of sodium concentration in the urine to that in the plasma (U:P) increased markedly during and after the infusion given during administration of SC8109. This was true in all cases although the values were quantitatively different in each patient.

From other studies of the chronic administration of SC8109 and of a similar lactone (SC9420) the maximum effect upon sodium excretion has been found to occur on the third day and to level off thereafter in most patients. Likewise, a decrease in potassium intake has been observed to reduce the natriuresis.[10] Such would be true on the day of infusion when the patient fasted in the

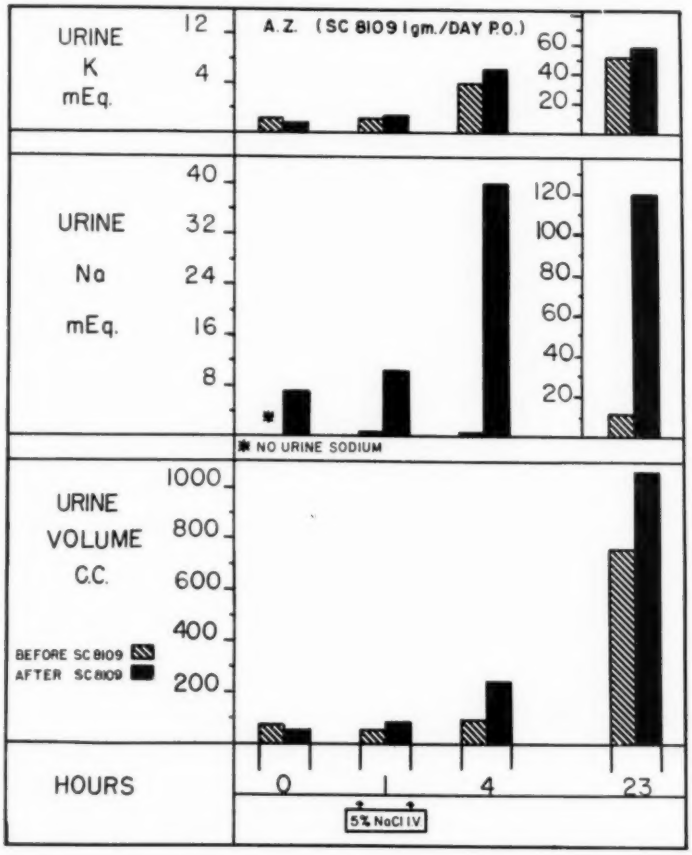

Fig. 1.—Changes in urinary excretion before and after saline infusion resulting from SC8109 administration; 0 hour refers to the urine collected for the hour immediately before infusion.

forenoon. With this information it was, therefore, deemed reasonable to determine the net increase in sodium excretion on the day of the infusion by subtracting the sodium excretion of the prior day from that of infusion days. From this could be calculated the per cent of the sodium load which was excreted during the control period and again after SC8109 administration. Comparison of the data for both infusions indicates a marked increase in net excretion in all patients after SC8109 (Table II).

Chronic Changes.—The urinary excretion of sodium increased markedly in all patients during the 4 day course of SC8109 (Table I, Fig. 2). The

maximal increase varied widely but ranged from 80 to 160 mEq. of sodium per day. An increase in sodium diuresis was observed with an increased dose of SC8109 in the 1 patient so studied (Fig. 3). In all studies urinary sodium fell to control levels within 48 hours after withdrawal of the drug. Potassium excretion was little affected by the drug, although in 2 patients (A. Z. and E. F.)

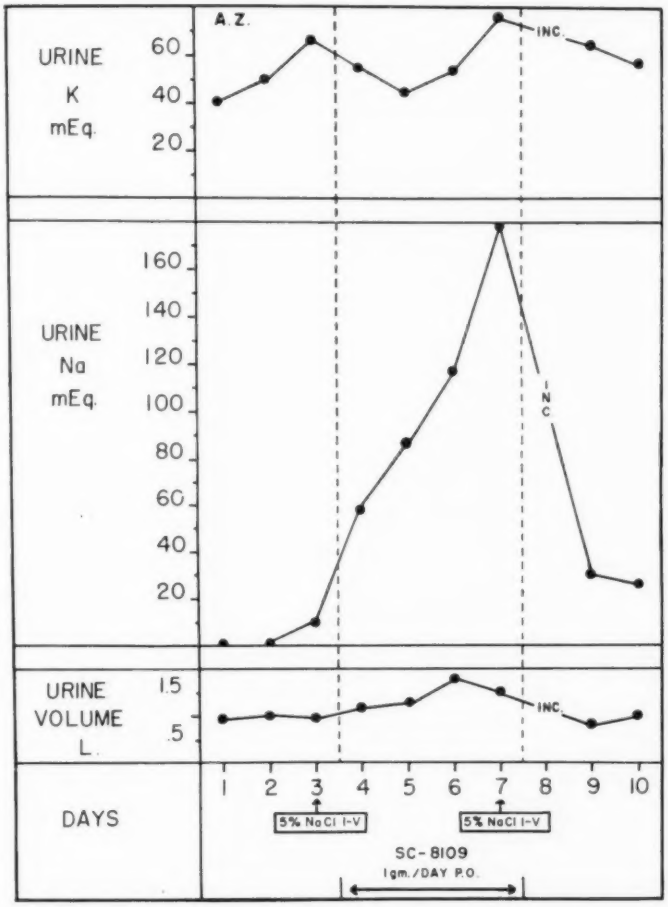

Fig. 2.—Daily urinary excretion changes during study. (*inc.* = Incomplete collection.)

there seemed to be some decrease during drug administration. This change is small and cannot be interpreted in a study of this size. Daily urine volume was not markedly affected by the SC8109 but only B. C. and E. F. had constant fluid intakes during the course of this investigation.

The urinary sodium : potassium ratio rose sharply in all cases during the period of drug administration (Fig. 4). This change, while striking, was qualitatively different in all patients and did not seem to be significantly affected by the hypertonic saline infusion.

DISCUSSION

In the normal individual it has been demonstrated that after an infusion of hypertonic saline a rapid diuresis ensues which is maximal in about 1 hour.[11-13] The glomerular filtration rate in such individuals rises coincident with diuresis but returns to normal before the diuresis has subsided. In patients with cirrhosis and ascites, however, hypertonic saline infusion has been found by Goodyer

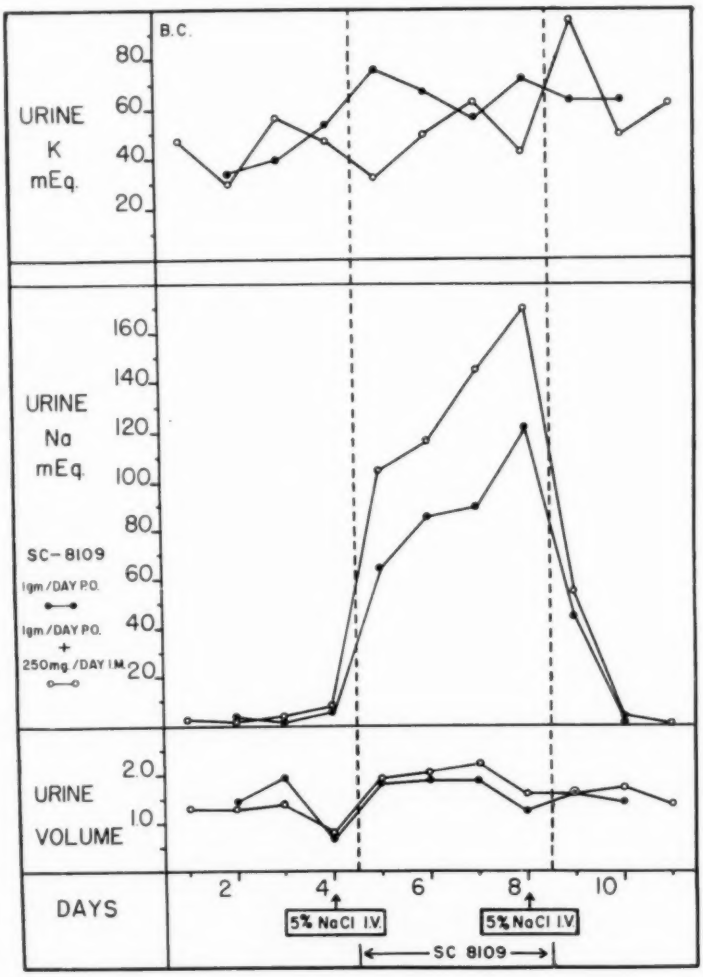

Fig. 3.—Daily urinary excretion changes during 2 studies in B. C. Note increase in changes with larger dose.

and co-workers[4] to produce a lesser increase in urine flow than in normal individuals and much less natriuresis. Thus, the patients with ascites excreted only 0.2 to 10 per cent of the sodium load while normal subjects and those without ascites excreted 12 to 54 per cent during the 6 to 8 hour test period. In our studies similar failure to excrete sodium was observed but after administration of the steroidal lactone all were able to excrete quantities of

sodium (20 to 45 per cent of infused load) which were in the normal range. In the report cited, furthermore, ability to concentrate sodium was likewise decreased in the patients with cirrhosis, none of whom exhibited urine:plasma ratios which exceeded unity, whereas this was achieved by all control subjects after infusion. Potassium excretion during the hypertonic saline infusion, however, was not markedly different in cirrhotic patients as compared to control subjects. An increase occurred coincidental with the natriuresis but was not consistently related to it.

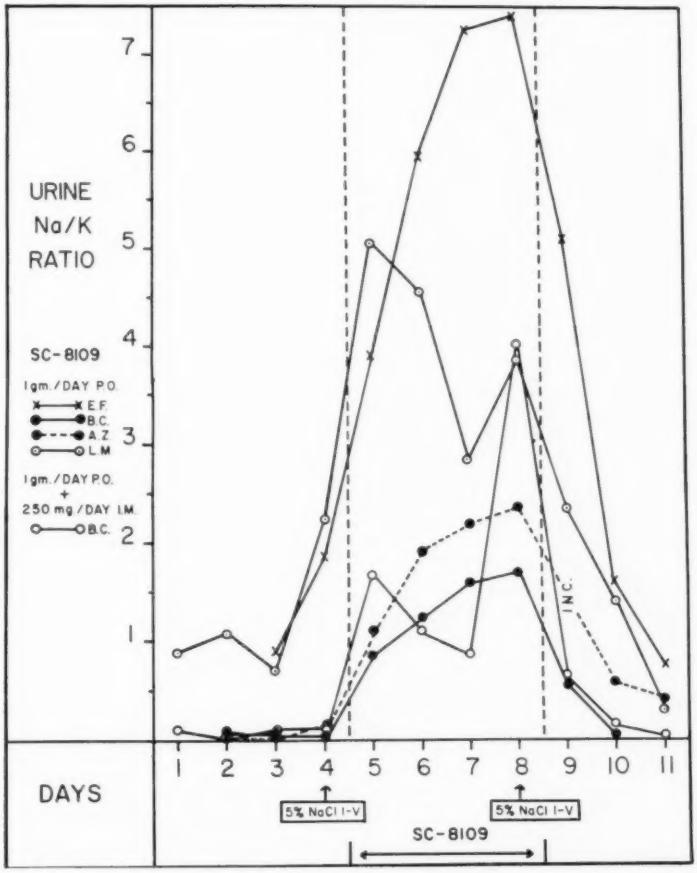

Fig. 4.—Changes in urine Na:K ratio during SC-8109 administration.

The response to hypertonic saline in our patients with ascites before administration of the spirolactone compound was similar to that observed in other studies and revealed a marked defect in sodium excretion. The response to the saline load after administration of SC8109, however, was quite different. At this time they were able to excrete significantly more sodium and more water but had little change in potassium excretion. Ability to concentrate sodium was enhanced as shown by the marked increase in urine:plasma sodium ratio. In some patients this exceeded unity after the second infusion thus

resembling the response seen in the normal control subjects. In order to explain these results we must assume that the final effect of the drug is a change in tubular function. If there was only an increase in glomerular filtration occurring, the urine concentration of sodium following each infusion should be similar and any increased sodium excretion should be due to an increase in total urine volume. The marked increase in urine sodium concentration and the decrease in urine potassium concentration, both in excess of the urine volume alteration, indicate that the predominant effect of SC8109 was altered tubular reabsorption. These findings are in accord with those of Wiggins and associates[14] who found no change in glomerular filtration when SC8109 was given to normal individuals who were receiving low sodium diets.

TABLE II. EFFECT OF SC8109 UPON NET SODIUM EXCRETION AFTER HYPERTONIC SALINE LOAD

PATIENT	URINE Na (mEq./day) BEFORE INFUSION	INFUSION DAY	DIFFERENCE	mEq. Na INFUSED	% EXCRETION OF Na LOAD IN 24 HOURS
E. F. control	52.2	82.8	30.6	177.5	17.3
after SC8109	155.0	235.2	80.2	177.5	45.3
B. C. (1) control	1.4	3.2	1.8	129.3	1.4
after SC8109	90.3	122.5	32.2	129.3	24.9
B. C. (2) control	2.9	7.2	4.3	129.3	3.3
after SC8109	144.0	171.7	27.7	129.3	21.4
A. Z. control	0.0	11.4	11.4	156.8	7.3
after SC8109	116.4	177.3	60.9	156.8	38.7
L. M. control	4.3	15.5	11.2	156.8	7.1
after SC8109	24.8	57.2	32.4	156.8	20.7

As noted previously, potassium excretion usually increases during hypertonic saline infusion.[4, 12] This is associated with an increased concentration of potassium in the urine. In 4 of 5 studies, however, the administration of SC8109 resulted in a lesser rise in urinary potassium concentration during saline infusion than was observed with the pretherapy infusion. This observation plus the remarkable rise in urinary Na:K ratio is compatible with the hypothesis that SC8109 is an antagonist of aldosterone.

The changes observed were qualitatively similar in all patients although quantitatively different as demonstrated by the variation in urinary sodium:potassium ratios. All patients had a significant sodium diuresis with little change in potassium excretion during the 4 days of administration of SC8109.

In view of the individual variation in response these quantitative differences might be due to variation in levels of circulating aldosterone from patient to patient. Thus, if more of the drug were given to a patient the magnitude of urinary sodium and potassium change should increase. This was attempted in 1 patient (B. C.) with resultant increased chronic sodium diuresis and some decrease in potassium excretion. The acute response during the hypertonic saline infusion, however, was not greater in respect to sodium diuresis, although potassium excretion was less; thus, a greater increase in Na:K ratio

occurred. Obviously, no clear-cut conclusions as to the effect of larger doses of SC8109 are possible from this single experiment.

The relative importance of aldosterone as opposed to other factors which affect renal function in the failure of the cirrhotic patient to excrete hypertonic saline cannot be elucidated from these studies. It appears, however, that reducing the effect of aldosterone upon the renal tubule produces a normal sodium excretory response in such patients.

SUMMARY

The effect of SC8109, a steroidal lactone reported to be an aldosterone antagonist, on the natriuretic ability of patients with cirrhosis and ascites has been studied by the use of hypertonic saline infusions.

Administration of this compound in 5 studies in 4 patients resulted in a marked natriuresis and a less marked water diuresis both during and after the infusion. Urinary potassium concentration was usually decreased during the 4 hours following infusion after administration of the lactone. The urine:plasma sodium ratio during the infusion increased markedly as did the urine Na:K ratio.

The daily urinary sodium excretion increased markedly during drug administration but neither daily potassium excretion nor urine volume were affected consistently.

The failure of patients with cirrhosis and ascites to excrete a sodium load has been confirmed. The administration of SC8109 caused a reversal of this response toward normal. The data are consistent with the hypothesis that the difficulty in sodium excretion in such patients may be related to the presence of aldosterone in high concentrations and that the natriuretic effect of this steroidal lactone may be due to inhibition of the effect of aldosterone at the renal tubule.

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REFERENCES

1. Falcon, W. W., Eckhardt, R. D., Cooper, A. M., and Davidson, C. S.: The Effect of Human Serum Albumin, Mercurial Diuretics, and a Low Sodium Diet on Sodium Excretion in Patients With Cirrhosis of the Liver, J. Clin. Invest. **28**: 595, 1949.
2. Eisenmenger, W. V., Blondheim, S. H., Bongiovani, A. M., and Kunkle, H. G.: Electrolyte Studies on Patients With Cirrhosis of the Liver, J. Clin. Invest. **29**: 1491, 1950.
3. Gabuzda, G. J., Jr., Traeger, H. S., and Davidson, C. S.: Hepatic Cirrhosis, Factors Contributing to the Failure to Excrete Urinary Sodium During the Accumulation of Ascites and Edema, J. Clin. Invest. **29**: 814, 1950.
4. Goodyer, A. V. N., Relman, A. S., Laurason, F. D., and Epstein, F. J.: Salt Retention in Cirrhosis of the Liver, J. Clin. Invest. **29**: 973, 1950.
5. Papper, S.: The Role of the Kidney in Laennec's Cirrhosis of the Liver, Medicine **37**: 299, 1958.
6. Luetscher, J. A., Jr., and Johnson, B. B.: Observations on the Sodium Retaining Corticoid in the Urine of Children and Adults in Relation to Sodium Balance and Edema, J. Clin. Invest. **33**: 1441, 1954.
7. Axelrod, B. V., Cates, J. E., Johnson, B. B., and Luetscher, J. A., Jr.: Aldosterone in Urine of Normal Man and of Patients With Oedema, Brit. M. J. **1**: 196, 1955.
8. Kagawa, C. M., Cella, J. A., and Van Arman, C. G.: Action of New Steroids in Blocking Effects of Aldosterone and Deoxycorticosterone on Salt, Science **126**: 1015, 1957.

9. Liddle, G. W.: Sodium Diuresis Induced by Steroidal Antagonists, Science **126**: 1016, 1957.
10. Taylor, F. F., and Faloon, W. W.: The Role of Potassium in the Natriuretic Response to a Steroidal Lactone (SC9420), J. Clin. Endocrinol. **29**: 1683, 1959.
11. Dean, R. F. A., and McCance, R. A.: The Renal Response of Infants and Adults to the Administration of Hypertonic Solutions of Sodium Chloride and Urea, J. Physiol. **109**: 81, 1949.
12. Papper, S., Saxon, L., Rosenbaum, J. D., and Cohen, H. W.: The Effects of Isotonic and Hypertonic Salt Solutions on the Renal Excretion of Sodium, J. LAB. & CLIN. MED. **47**: 776, 1956.
13. Wesson, Laurence G., Jr.: Glomerular and Tubular Factors in the Renal Excretion of Sodium Chloride, Medicine **36**: 281, 1957.
14. Wiggins, R. A., Hutchin, M. E., Carbone, J. V., and Doolan, P. D.: Effect of Spirolactone SC8109 on Renal Function in Normal Human Subjects, Proc. Soc. Exper. Biol. & Med. **100**: 625, 1959.

AN EVALUATION OF THE URINE HYDROLYSIS TEST FOR PRIMITIVE WHITE BLOOD CELL DIFFERENTIATION

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ACCURATE identification of primitive leukemic white blood cells in peripheral blood and bone marrow is necessary before the most effective therapy can be instituted. Even after using a variety of technical aids it is often difficult to classify the cell type. Any new method which proposes to facilitate the study and identification of early cell types can be expected to be of interest. Such it is with the technique of treating blood films with urine to produce a differentiation of myelocytic cells from other leukocytes.

Urine hydrolysis was first proposed by Brachet[1] and has been explored more recently by others.[2-4, 6] The test involves the exposure of bone marrow or peripheral blood films, previously fixed in methyl alcohol, to the action of as yet unidentified substances (probably enzymes) in urine. Nuclei of myelocytic cells are "lysed" while lymphocytic and monocytic nuclei remain relatively intact. Such cells appear after staining as though the nuclear material has been completely removed leaving only vestigial chromatin (Fig. 1). The usefulness of this technique as an aid in the identification of primitive white blood cells depends largely on the consistency of the reaction to all the cells of the myelocytic series. One study relating the age of the granulocyte and its susceptibility to hydrolysis by urine was made by Perkowska.[2] The purpose of the current study was to establish standardized conditions for carrying out the test in preparation for the more detailed observations on application of the test to the cells of the granulocytic series in various stages in their development.

MATERIALS AND METHODS

Urine samples were obtained from normal individuals and patients with a variety of diseases. An attempt was made to use first morning specimens when possible.

Enzymes and Substrates.—Desoxyribonucleic acid, ribonucleic acid, and the corresponding enzymes were obtained commercially.*

Phosphate buffer, pH 7.4, was made from 0.01M Na_2HPO_4, 0.1M NaH_2PO_4.

Buffered saline was made from 0.85 per cent NaCl in phosphate buffer, pH 7.4.

Enzymes used were: desoxyribonuclease (DNAse), 5 mg. (± 0.2 mg.) in 25 ml. phosphate saline to final concentration 0.2 mg. per milliliter; and ribonuclease (RNAse), 5 mg. (± 0.2 mg.) in 25 ml. phosphate saline to concentration of 0.2 mg. per milliliter.

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Blood Films.—Bone marrow and peripheral blood films are made in similar manner on glass slides. Films are allowed to air-dry thoroughly and are then methanol fixed for 15 minutes, and are air-dried again before use.

Urine.—The pH is determined on the unaltered specimen. Changes in pH are made by the addition of a weak solution of sodium hydroxide or hydrochloric acid. Tests have been run at pH 5.6, 7, and 7.4.

Hot Water Incubator.—Urine and enzyme solutions are allowed to come to 60° C. (± 2°) before the films are immersed in the solutions.

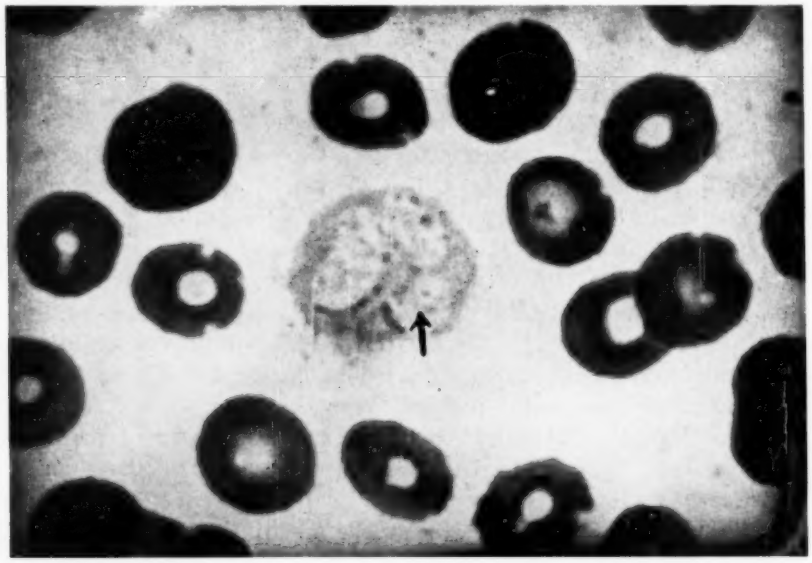

Fig. 1.—Single segmented neutrophil treated with normal urine showing complete lysis of nucleus.

PROCEDURE

Prepared urine or enzyme solutions in Coplin jars are placed in the 60° C. water bath. The temperature of the solutions is allowed to rise to 60° C. (± 2°). Prepared films, properly marked, are placed in the preheated solutions for complete coverage of blood film. Films remain for designated periods of time (usually 10 or 15, 20 or 30, 45, or 60 minutes). The film is then removed and is put into another Coplin jar. Tap water is run continuously over the films for 1 to 2 minutes. Films are air-dried and stained with the desired stain. A control untreated film is stained at the same time as the test films.

Stained films are examined microscopically to determine the degree of hydrolysis of the cell nuclei. The phase microscope has proved useful in certain cases.

With limited amounts of enzyme solutions, we have used the following technique: Petri dishes are floated on the 60° C. water with their cover dishes. A strip of filter paper slightly smaller than the size of the glass slides is dipped in the 60° C. water and placed flat in the dish. The enzyme solution is heated in a test tube for approximately 5 minutes (i.e., until solution temperature is 60° C.) just before use on the film. The prepared blood film is placed flat on the warmed wet paper strip; the enzyme solution is put on the film with a pipette until covered. Care must be used to prevent the enzyme solutions from draining off the slides during incubation. The Petri dish cover is now replaced and incubation timing is started. At the end of the incubation period, the films are removed from the floating

Petri dish, the enzyme solution is drained off, and films are placed in a Coplin jar to be washed with running tap water. Films are then handled in the same manner as in the preceding procedure.

RESULTS

Preliminary experiments in which peripheral blood films were subjected to the action of urine demonstrated a selective action of the urine on the various cells of the different leukocytic series. However, it soon became apparent that this activity was not constant and that a number of variables affected the action of the urine on the exposed cells. It was observed that certain urine specimens acted only slightly or not at all on the blood films and there seemed to be a variation in the resistance of cells in films from some pathologic blood.

In order to understand the factors which influenced the test, the following observations were made:

1. *Influence of Temperature and Time.*—An experiment was designed to determine optimum temperature and time at which the most selective action of the urine on various types of cells occurred (Table I, Part I). The results indicate inhibition of the activity of the urine at temperatures of 5 and 20° C. for the shorter periods of time with a slight increase in activity during the 45 and 60 minute incubation. Maximum effect of the urine occurred at a temperature of 60° C., at which temperature 15 minutes' incubation provided adequate time for complete dissolution of the nuclear material of the neutrophil with negligible effect on the lymphocytes and monocytes. Incubation at 80° C. resulted in a decrease in lysis accompanied by an increase in diffusion of nuclear material into the immediate cell area, whereas 100° C. completely inhibited the typical lytic type reaction and resulted in a "moth eaten" appearance to the segmented cells unlike that seen at lower temperatures.

2. *The Effect of pH.*—It was noted that a variation in the pH of normal urine resulted in a differential effect on the blood films; consequently, the above experiment was repeated on urine specimens which were adjusted to pH 7.4 (Table I, Part II). There was an accentuation of the activity of the urine in lysing cell nuclei of the granulocytes at each temperature within the active range (20 to 80° C.) with little or no additional action on cells of the mononuclear series. Additional experiments on the effect of pH demonstrated that the lysis of the nucleus was dependent to a large extent on this factor. Thus a urine specimen with a pH of 5 to 6 showed only minimal lytic effect on the nuclear contents, whereas adjustment of the same urine specimen to a neutral or slightly alkaline pH resulted in maximum activity. The increase in lytic activity resulting from pH adjustment enabled the time of incubation to be shortened in many cases. Thus 5 or 10 minutes' incubation proved satisfactory for most urine samples.

3. *Use of Salt and Enzyme Preparations.*—The nature of the action of urine on granulocytic nuclei has not been determined. Thoma[3] attributes the effect to a RNAse complex of the urine, whereas more recently Sprague and co-workers,[4] using crystalline RNAse, reported no change comparable to that suggested by Thoma. They suggested that the effect of urine may be due to

TABLE I. INFLUENCE OF TIME, TEMPERATURE, AND pH ON THE ACTIVITY OF NORMAL URINE ON NORMAL BLOOD FILMS

TEMPERATURE (° C.)	TIME OF INCUBATION (MINUTES)	PART I, pH UNADJUSTED:		PART II, pH ADJUSTED:	
		POLYMORPHONUCLEAR	MONONUCLEAR	POLYMORPHONUCLEAR	MONONUCLEAR
5	10	None	None	± with diffusion	None
	15	None	None	± with diffusion	None
	30	±	None	± with diffusion	None
	45	±	None		
	60	+ with diffusion	None		
20	10	None	None	± with diffusion	None
	15	None	None	± with diffusion	None
	30	+ with diffusion	None	+ with diffusion	None
	45	+ with diffusion	None		
	60	+ +	None		
40	10			+ + + +	±
	15	+ + +	±	+ + + +	±
	30	+ + + − + + + +	±	+ + + +	± − + +
	45	+ + + − + + + +	±		
	60	+ + + +	+ + + − + + + +		
60	10			+ + + +	±
	15	+ + + +	±	+ + + +	±
	30	+ + + +	+	+ + + +	±
	45	+ + + +	+		
	60	+ + + +	+ − + +		
80	10			± − ++ with diffusion	± with diffusion
	15	+ with diffusion	±	± − ++ with diffusion	± with diffusion
	30	++ with diffusion	±	± − ++ with diffusion	± with diffusion
	45	++ with diffusion	±		
	60	++ with diffusion	±		
100	10				
	15	+*	±		
	30	+*	±		
	45	++*	±		
	60	++*	±		

None, Represents no effect on nucleus.

±, Represents an intact nuclear wall with nucleus staining lighter and a slight homogenization of the chromatin.

+, Represents an intact, condensed, nuclear wall; nuclear staining is still lighter and the chromatin smoother.

++, Represents a condensed, intact nuclear wall; nucleus staining with traces of blue and unstained when lysis is complete; approximately one half the chromatin remaining.

+++, Represents a roughly intact nuclear wall; the nuclear wall and chromatin now stains a deep pink blue; remaining traces of chromatin appear aggregated and condensed.

++++, Represents an indistinct nuclear wall with faint residual condensed chromatin network, both staining slightly pink in an empty nuclear [illegible].

Diffusion, Indicates a "leaking" of nuclear material from the nucleus into adjacent areas.

*Nuclei of neutrophils at this temperature present a punched-out or moth eaten appearance, as opposed to the typical homogenous lytic effect.

extraction of nucleoprotein from the cell by the dissolved salt in the urine with the possibility that an additional role be ascribed to the action of proteolytic enzymes present in the substrate.

TABLE II. EFFECT OF VARIOUS CONCENTRATIONS OF NaCl IN SOLUTION ON NORMAL BLOOD FILMS AT 60° C.

SALT CONCENTRATION	INCUBATION (MINUTES)	RESULTS	
		POLYMORPHONUCLEAR	MONONUCLEAR
Distilled H_2O	10	++ hydrolysis with nuclear shattering	±
0.017M	10	+++ with nuclear shattering and diffusion	+ with nuclear shattering and diffusion
	20	+++ with nuclear shattering and diffusion	+ and ++ with nuclear shattering and diffusion
0.068M	10	+++ with nuclear shattering and diffusion	0 – + with diffusion
	20	+++ with nuclear shattering and diffusion	± and + with diffusion
0.085M	10	++ and ++++ with diffusion and nuclear shattering	± with diffusion
	20	++ and ++++ with diffusion and nuclear shattering	± and + with diffusion
0.145M	10	+++ with diffusion	± with diffusion
	20	+++ and ++++ with diffusion	± with diffusion
0.256M	10	++ and +++ with diffusion	± with diffusion
	20	++ and +++ with diffusion	+ with diffusion
0.342M	10	+++ with diffusion	+ with diffusion
	20	+++ with diffusion	+ with diffusion

None, No nuclear effects.

±, Indicates a faint nuclear stain, nuclear wall slightly condensed, nuclear chromatin slightly smoother.

+, Indicates more pronounced nuclear wall condensation with nuclear chromatin smoother; nuclear stain is less blue and more a muddy pink.

++, Indicates about one half of nuclear chromatin remaining within the nucleus and nuclear wall roughly outlined. Staining characteristic is a deep muddy pink with a bluish tinge.

+++, Indicates a rough nuclear wall outline with most of nuclear chromatin lysed. The remaining chromatin stains a muddy pink with a faint bluish tinge.

++++, Indicates complete chromatin lysis; nuclear wall condensation which remains stains faint pink.

Diffusion, Indicates a leaking of the nuclear material from within the nuclear walls into the surrounding area of cytoplasm and then into the adjacent cell area.

Nuclear shattering, Indicates that the nucleus appears to have exploded and the nuclear material has been forced outward in an exploded pattern.

TABLE III. COMPARISON OF THE ACTIVITIES OF RNAse AND DNAse IN PHOSPHATE BUFFER AND DISTILLED H_2O ON THE NUCLEI OF NORMAL BLOOD FILMS

ENZYME	DILUENT	RESULTS	
		POLYMORPHONUCLEAR	MONONUCLEAR
DNAse	H_2O	+ – +++	+ – ++
	Buffered 0.85% saline	++++	++ – +++
RNAse	H_2O	+ (stain affinity greatly reduced)	± – + (stain affinity greatly reduced)
	Buffered 0.85% saline	+ – ++ with diffusion	0 – + with slight diffusion

To further study urine substances responsible for the lytic effect on the white blood cells the following was done:

The effect of varying concentrations of saline solutions on prepared normal blood films was determined. These salt solutions contained, respectively, 0.017M,

0.068M, 0.085M, 0.145M, 0.256M, and 0.342M NaCl. Distilled H_2O was included for comparison. Incubation of the blood films was carried out at 60° C. for 10 and 20 minutes after which they were stained with Wright's stain. The pH of the salt solutions was in the range of 6.5 to 7.0. The results are shown in Table II.

Examination of the stained films showed partial dissolution of the nuclear contents of the cell; however, most of the material appeared to have diffused

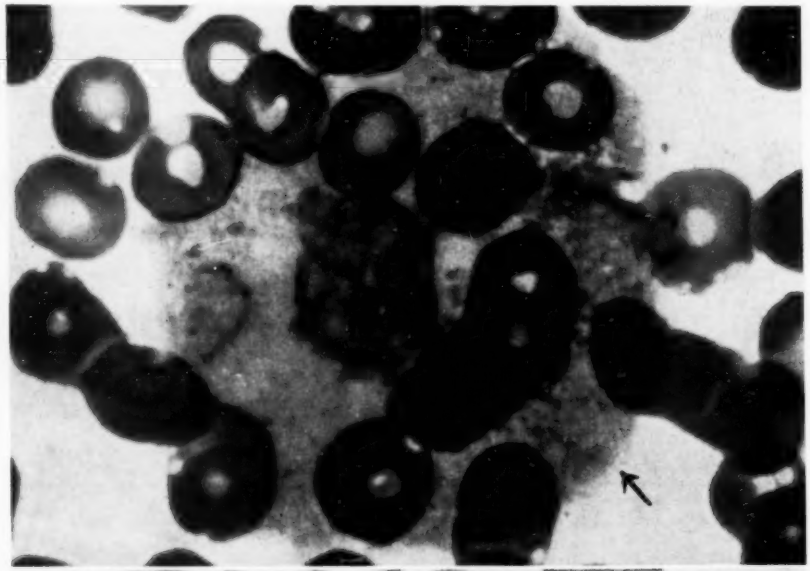

Fig. 2.

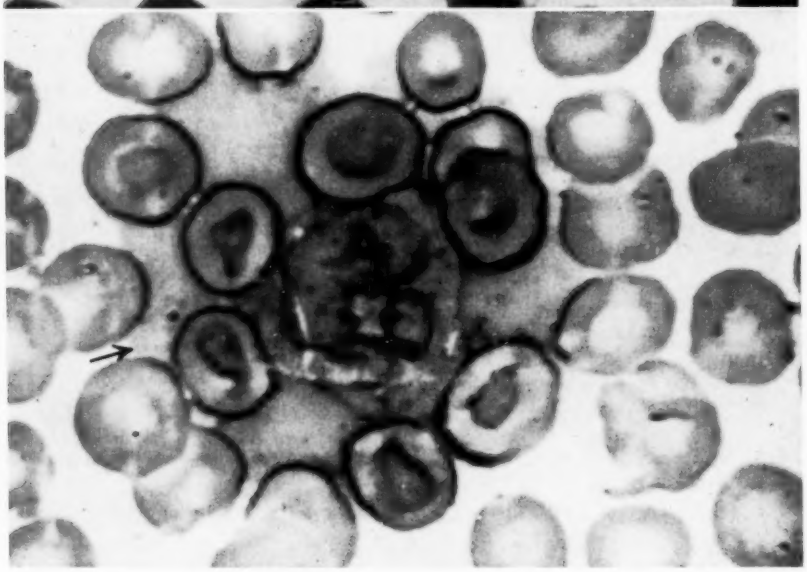

Fig. 3.

Fig. 2.—Single segmented neutrophil, treated with 0.68M NaCl. The arrow shows edge of the diffusion of the ruptured nucleus.

Fig. 3.—Single segmented neutrophil, treated with 7.4 buffered saline solution; arrow shows edge of the diffusion of the ruptured nucleus.

into the area immediately surrounding the cells, producing a diffuse blue halo effect when stained with Wright's stain (Figs. 2 and 3). This material was subsequently shown to react positively in the Feulgen reaction. The affected nucleus did not resemble that which is seen after exposure to urine. Most of the cells exposed to the salt solutions showed rupture of the nuclear membrane and cell wall. It appeared that this type of partial lysis is the result of simple dissolution of the nucleoprotein with the salt solution acting as solvent. This was particularly evident in concentrations of 0.085M and 0.145M. In

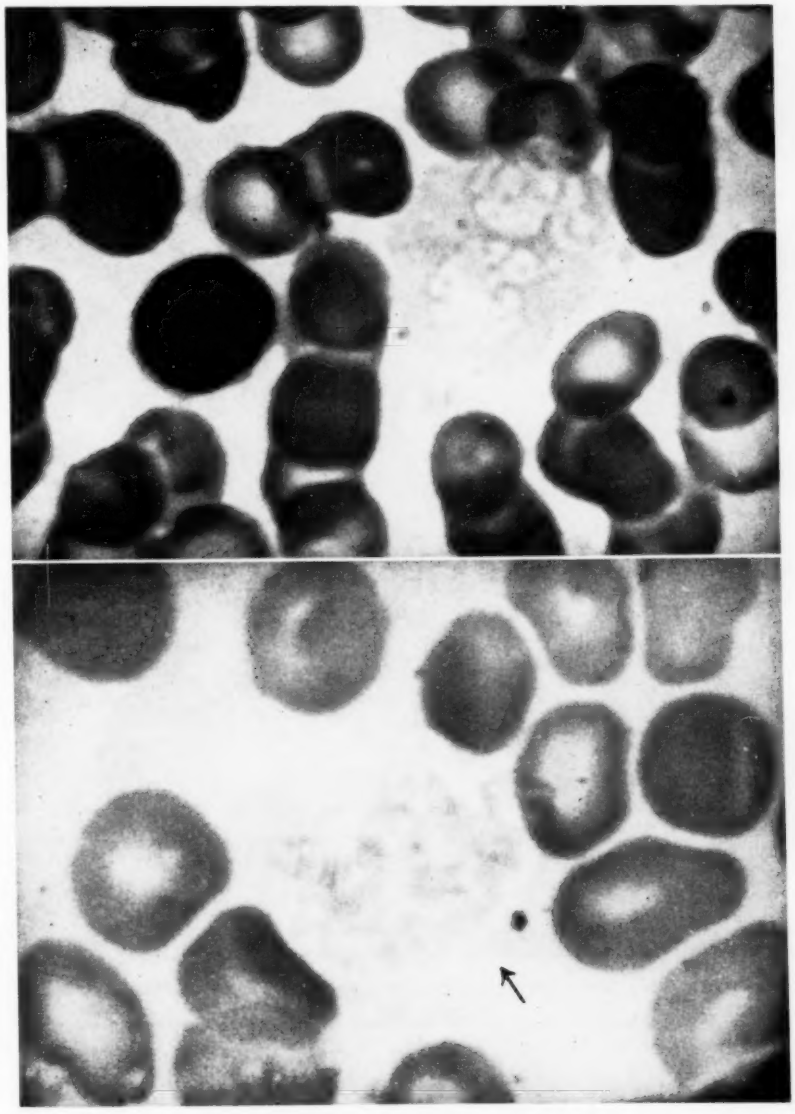

Fig. 4.

Fig. 5.

Fig. 4.—Lymphocyte (left) and segmented neutrophil (right) treated with DNAse in saline showing complete lysis of the neutrophil nucleus.

Fig. 5.—Single segmented neutrophil treated with DNAse in water showing incomplete hydrolysis.

order to compare the action of urine and saline solution to the activities of purified enzyme preparations, solutions which contained 20 mg. per cent crystalline RNAse and DNAse were prepared in phosphate buffer saline, pH 7.4, and in distilled water and were incubated with normal blood films. Table

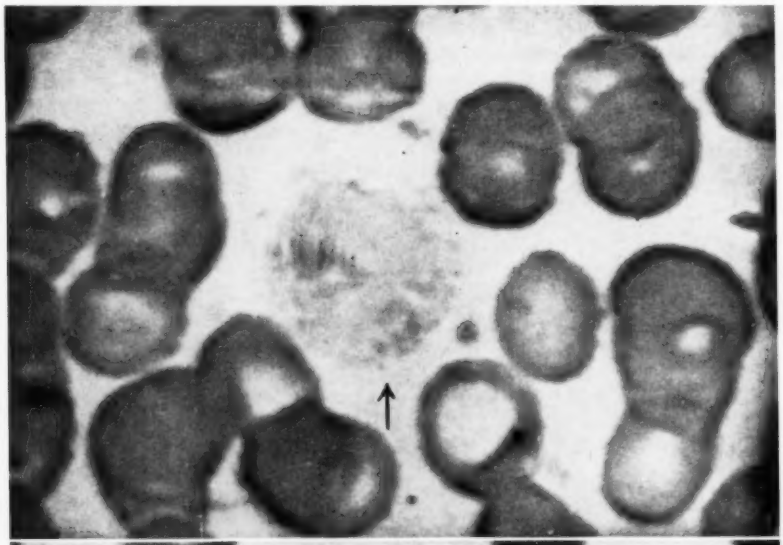

Fig. 6.

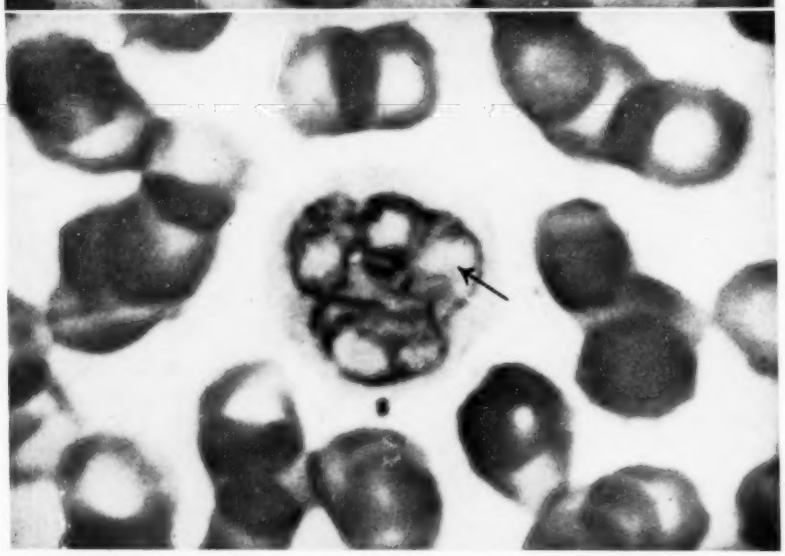

Fig. 7.

Fig. 6.—Single segmented neutrophil treated with RNAse in H_2O showing decreased affinity for staining.

Fig. 7.—Single segmented neutrophil, treated with RNAse in saline, showing incomplete lysis of nucleus.

III summarizes the results of this experiment. A comparison of these results readily shows that the combination of DNAse in phophate saline, pH 7.4, was the only one in which results were comparable to those obtained with normal

urine (Fig. 4). Cells in blood films exposed to the same concentration of DNAse in distilled H_2O showed a diminished staining ability with vacuolation of the nucleus and cytoplasm (Fig. 5).

RNAse in distilled water resulted in a loss of staining ability for the leukocytes of all series (Fig. 6). In many cases a pseudolysis of the nucleus occurred unlike that produced by the urine and usually accompanied by diffusion of the nuclear material into the surrounding area.

When RNAse in phosphate saline was used, the nuclei of the granulocytic series again showed an incomplete lysis with condensation of the nuclear material (Fig. 7). In neither RNAse in saline nor RNAse in water was there significant effect on the mononuclear cells. These results with purified RNAse are in accord with those of Thoma[5] who found only unpurified enzymes capable of duplicating the effect of urine hydrolysis.

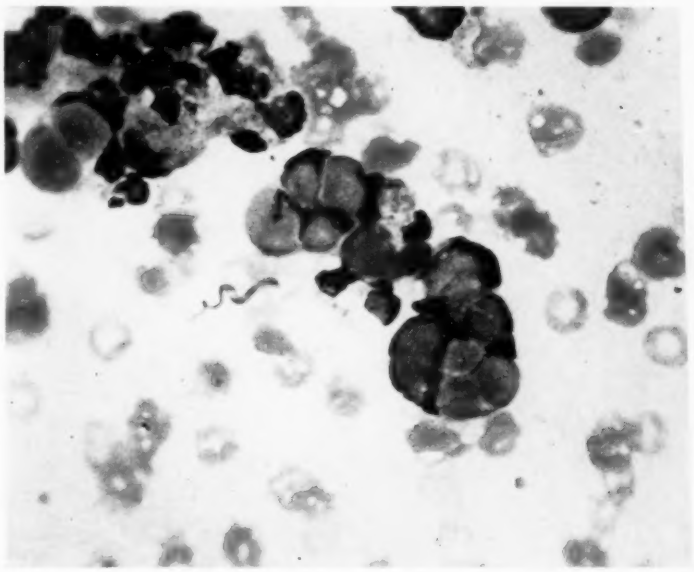

Fig. 8.—Control L.E. cells.

4. *Clinical Application of Urine Test.*—In leukemia of various types application of the urine test to blood films showed a close correlation with the predicted action, that is lysis of myelocytic nuclei. In only one instance (Patient MC) was there a discrepancy between the interpretation via the usual cytologic methods and the urine hydrolysis technique. These differences may have resulted (1) from prior treatment of the patient which resulted in a change in the composition of the cells, (2) as the direct result of the therapeutic chemical, or (3) misinterpretation of the cell type.

Table IV shows the results of the urine hydrolysis test when applied to this group of patients. In most instances, the test was carried out at 56 to 60° C. for 10 to 15 minutes, using urine adjusted to a pH of 7.0.

TABLE IV. EFFECT OF URINE (pH 7.0) ON WHITE BLOOD CELL DIFFERENTIAL

PATIENT	SOURCE	CLINICAL DIAGNOSIS		POLYMORPHONUCLEAR							MONONUCLEAR				TOTAL W.B.C.
				PMN	BANDS	META.	MYEL.	BLAST.	BASO.	EOSIN.	LYMPH.	BLAST.	MONO.	BLAST.	
JH	Peripheral	Acute lymphocytic	Differential	4	2						6	88			2,900
			Degree hydrolysis	++++	++++						0	0			
	Bone Marrow	Acute lymphocytic	Differential				1		2	2		93			
			Degree hydrolysis				++++		0	0		0			
TED	Peripheral	Chronic lymphocytic	Differential	11							88		1		28,500
			Degree hydrolysis	++++							0		±		
LD	Peripheral	Acute lymphocytic	Differential	39						1	59		1		5,565
			Degree hydrolysis	++++						0	0		0		
CG	Peripheral	Acute lymphocytic	Differential	1							99				735,500
			Degree hydrolysis	++++							±				
CR	Peripheral	Chronic lymphocytic	Differential	1							98		1		214,000
			Degree hydrolysis	++++							0		0		
MG	Peripheral	Monocytic	Differential	28	5	4	1				3	53	6		94,500
			Degree hydrolysis	++++	++++	++++	++++				0	±	±		
GB	Peripheral	Monocytic	Differential	13	2	5	2				12		11	55	55,500
			Degree hydrolysis	++++	++++	++++	++++				0		0	0	
EA	Peripheral	Monocytic (Naegeli type)	Differential	12	6	2					74		6		900
			Degree hydrolysis	++++	++++	++++					0		0		
	Bone Marrow	Monocytic (Naegeli type)	Differential	9	10	12	25	3			11		20	10	
			Degree hydrolysis	++++	++++	++++	++++	++++			0		±	±	
MB	Peripheral	Acute monocytic	Differential	1							35		64		6,100
			Degree hydrolysis	++++							0		0		
	Bone Marrow	Acute monocytic	Differential*				7						90		
			Degree hydrolysis				++++						0		
JB	Peripheral	Acute lymphocytic	Differential	31	3						55	10	1		5,350
			Degree	++++	++++						0	0	0		

JB	Peripheral	Acute lymphocytic	Differential	31	3						55	10	1		5,350
			Degree hydrolysis	++++	++++						0	0	0		
[illegible]	[illegible]	Acute myelocytic	Differential	16	17	4	21	34	1		5		2		108,000
			Degree hydrolysis	++++	++++	++++	++++	++++	0		0		0		
MC	Peripheral	Acute myelocytic	Differential	1				99							99,500
			Degree hydrolysis	++++				0							
WD	Peripheral	Chronic myelocytic	Differential	39	8	4	21	1			23		4		24,000
			Degree hydrolysis	++++	++++	++++	±	0			0		0		
	Bone Marrow	Chronic myelocytic	Differential	11	3	5	55	10		13	1		2		
			Degree hydrolysis	++++	++++	++++	++++	0		0	0		0		
FC	Peripheral	Chronic myelocytic	Differential	18	37	9	12	2	10	7	4		1		187,000
			Degree hydrolysis	++++	++++	++++	++++	++++	++++	±	±		±		
HN	Peripheral	Acute myelocytic	Differential	4	1	6	49	40							344,000
			Degree hydrolysis	++++	0	0	0	0							
	Bone Marrow	Acute myelocytic	Differential	1	1	2	90	6							
			Degree hydrolysis	++++	++++	0	0	0							
JM	Peripheral	Chronic myelocytic	Differential	50	8	12	7		2	3	3		15		25,500
			Degree hydrolysis	++++	++++	++++	++++		0	0	0		±		
CD	Peripheral	Chronic myelocytic	Differential	46	18	2	15	1	15	2	1				51,500
			Degree hydrolysis	++++	++++	++++	++++	++++	±	0	0		±		
DSS	Bone Marrow	Pernicious anemia	Differential†	24	20	20	4			2					
			Degree hydrolysis	++++	++++	++++	++++			+++					
MSW	Peripheral	Myelocytic	Differential	8							18		72	2	5,500
			Degree hydrolysis	++++							0		0	0	
	Bone Marrow	Myelocytic	Differential‡	15	10	10	38			20	1				
			Degree hydrolysis	++++	++++	±	±			0	0				

*Plasma cells 3 0.
†Phagocytes 30 ++++.
‡Phagocytes 6 0.

Exposure of lupus erythematosus (L.E.) cells to the action of urine presented a variable picture on different parts of the cell. Fig. 8 shows a control group of L.E. cells. Figs. 9 and 10 show 2 of the variations encountered. Fig. 9 demonstrates complete (++++) hydrolysis of the nucleus of the phagocytic neutrophil with only minimal (+) effect on the inclusion body. Fig. 10 shows complete hydrolysis of both the neutrophilic nucleus and the inclusion body. Still other tests resulted only in the inclusion body being hydrolyzed with no effect on the engulfing cell nucleus. These variations may represent differences

Fig. 9.

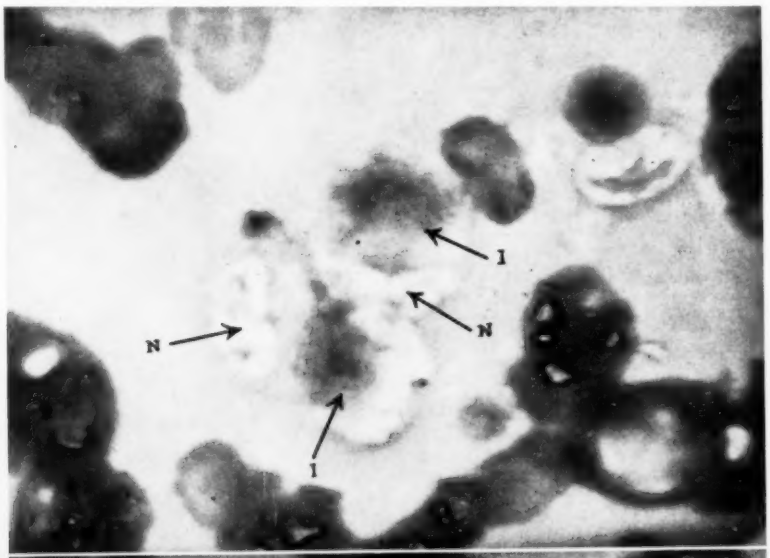

Fig. 10.

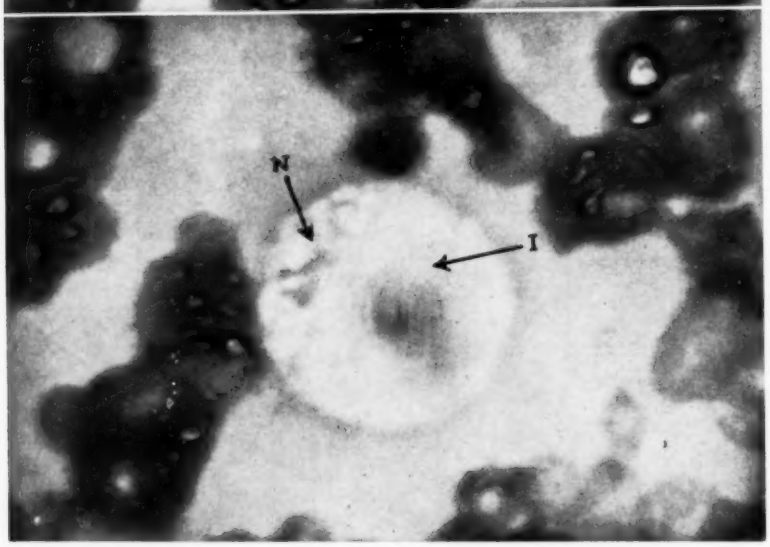

Fig. 9.—Three L.E. cells treated with normal urine showing complete or 4 plus hydrolysis of nucleus of engulfing neutrophil *(N)* and 1 plus hydrolysis of inclusion body *(I)*.

Fig. 10.—Single L.E. cell treated with normal urine; 4 plus hydrolysis of engulfing nucleus *(N)* and 4 plus hydrolysis of inclusion body *(I)*.

in the integrity of L.E. cells, or more probably a difference in the cell type from which the L.E. cell is derived. Thus, an inclusion body formed from a mononuclear cell might be expected to be more resistant to hydrolysis by urine than an inclusion body derived from a neutrophil if no change in the susceptibility of the nuclear material to hydrolysis occurs during its "depolymerization."

DISCUSSION

Urine hydrolysis of the leukocytic cells in peripheral blood and bone marrow films has been suggested as an aid in the differentiation of cell types. Difficulties are frequently encountered, particularly with primitive leukocytic cells. With the advent of new therapeutic materials which apparently have a relative "selective" action on the various cell types,* it has become increasingly more important that precise cell identification be made. There is every indication that the importance of accurate cell identification will become more acute in the future.

In our laboratory we have utilized a variety of means to aid in the identification of these cells which are more difficult to recognize; frequently a panel of tests is employed. These techniques include Wright's, supravital, and peroxidase staining, phase microscopy, tissue culture studies, and other specialized staining techniques. The hope that urine hydrolysis may be useful as an aid in the identification of the early blast cells led to the present investigation of the factors concerned with its use.

It is not clear what substances in urine are responsible for lysis of the cell nucleus. There have been vigorous proponents for both RNAse and DNAse as the active substance. Others have suggested a combination of proteolytic enzymes and at least one has presented evidence for the participation of the salt content of the urine. Evidence from the present study suggests participation of the factors mentioned. The possibility that a more or less highly purified enzyme preparation could produce more definitive information is acknowledged; however, only DNAse seemed to produce an effect comparable to that which occurred with urine. All other preparations in our hands produced only a pseudo-type lytic effect. As an example, exposing blood films to various saline concentrations resulted in an obvious dissolution of nuclear material which resembled, in some respects, the effect produced by urine hydrolysis; however in most cases, the effect was accompanied by a rupture of the wall of the nucleus at lower salt concentrations and by a diffusion of nuclear material into the area adjacent to the cell at every concentration of saline employed. The resulting halo effect when stained with Wright's stain and the reaction of the material to the Feulgen procedure point to dissolution of nuclear substance unlike the apparent enzymatic changes which occur with urine. This halo effect produced by saline solutions and other agents has not been observed in films exposed to the action of urine at 60° C. but was observed when the test was carried out at 5 and 80° C. and was probably due to the action of the salt in the urine with little participation of enzyme at these temperatures.

*Acute lymphocytic leukemia is most effectively controlled with the antifolics and 6-mercaptopurine; acute granulocytic with 6-mercaptopurine, the antifolics having no effect. Acute monocytic leukemia is affected by neither group.

Essentially, 3 factors, the pH and temperature of the urine and time, should be controlled in the use of urine hydrolysis for demonstrating a differential effect on the nucleus of white blood cells. The pH of the urine specimen should be determined initially and adjusted to approximate neutrality. Apparently, maximum differential hydrolysis of the nuclei occurs at a temperature approaching 60° C. At this temperature the time factor is reduced so that the desired results are obtained in 5 to 10 minutes in many cases.

Actual enzyme measurements are contemplated and should be of interest for determining the enzyme responsible for nuclear lysis. There have been indications from the present study that variations in enzyme content of urine specimens from individuals with various hematologic diseases and normal individuals may reflect the metabolic changes of the body during periods of change in condition and treatment. Theoretically the use of standardized enzyme preparations should facilitate the test; however, in preliminary attempts it has not been possible to supplant with assurance the urine as the hydrolyzing agent in the test routine.

Continued evaluation of the use of the test for cell identification is being made and will be considered more fully when sufficient information has been obtained.

SUMMARY

Conditions affecting the use of urine hydrolysis for the differentiation of white blood cells are discussed and findings indicate pH, temperature and time are the most important factors to be controlled.

Observations on the effect of purified enzyme preparations in an attempt to duplicate the effect of urine revealed that only desoxyribonuclease (DNAse) in buffered saline gave comparable results.

Application of the urine test to blood films from a variety of hematologic patients substantiated the validity of the differential action in lysing cells of the neutrophilic series. The selective action was apparent in the very young cells of this series and was judged of value as an aid to cell identification.

Additional studies concerning identification of the responsible enzymes and the relationship of the enzyme concentration in urine to pathologic conditions are in progress.

Exposure of lupus erythematosus (L.E.) cells to the action of urine presented a variable picture on different parts of the cell. These variations may represent a difference in the cell type from which the L.E. cell is derived.

REFERENCES

1. Brachet, J.: La localization des acides pentosenucleiques dans les tissus animaux et les oeufs d'Amphibiens en voie de developpement, Arch. biol., Paris **53**: 207, 1942.
2. Perkowska, E.: Relationship Between the Age of the Neutrophile and the Susceptibility of Its Nucleus to Hydrolysis With Urine Dialysate, Acta haemat. **17**: 247, 1957.
3. Thoma, K.: Über die Verwendung von Ribonuclease (Brachet-Test) in der hämatologischen Diagnostik, Acta haemat. **4**: 81, 1950.
4. Sprague, C. C., Green, R. T., and Carrera, A. E.: Differential Extraction of Nucleoprotein From Human Leukocytes, J. LAB. & CLIN. MED. **50**: 955, 1957.
5. Thoma, K.: Personal communication.
6. Aleksandrowicz, J., Urbanczyk, J., Ostrowska, A., and Sierko, J.: Further Research on the Activity of Ribonuclease in the Blood and Urine of Patients Suffering From Proliferative Hemocytopathia, Blood **13**: 652, 1958.

EFFECTS OF INFUSION OF MOLAR SOLUTIONS OF HYPERTONIC SODIUM LACTATE AND SODIUM CHLORIDE ON RENAL EXCRETION OF SODIUM AND WATER

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IN THE therapy of slow heart rates, cardiac arrest, and hyperpotassemia[1-4, 16] molar sodium lactate has been found to be of value in increasing cardiac rhythmicity. Furthermore, molar sodium lactate has been found to have therapeutic value in electrolyte imbalance associated with both renal and metabolic acidosis.[5-8]

It was noted that many patients with heart disease, often of a severe grade, were able to tolerate large doses of molar sodium lactate without the development of heart failure.[2, 15, 16] These observations prompted us to determine how the normal human kidney tolerates molar sodium lactate as compared to equimolar sodium chloride.

METHOD AND MATERIALS

Ten normal subjects without any evidence of renal, cardiac, or electrolyte abnormalities, between the ages of 19 and 36, were selected from the wards of the Philadelphia General Hospital. Ringer's solution was infused intravenously at a rate of 10 ml. per minute until a constant urine flow was achieved. The average volume of Ringer's infusion needed to achieve a constant diuresis was 2,650 ml. infused over a period of 4 hours. Urine was collected through an indwelling catheter at 10 minute intervals. Simultaneously, heparinized blood samples were drawn at the end of each collection period from the contralateral arm. After three 10 minute control periods, when both sodium and water excretion were constant, either one molar sodium lactate (6 patients) or one molar sodium chloride (4 patients) was substituted at the same rate of infusion. Urine and blood samples were taken at 10 minute intervals for 3 experimental periods. The urine and blood samples were analyzed for sodium, potassium, total solutes, and creatinine by methods previously described.[10, 17, 18]

RESULTS

The comparison of the effects of sodium lactate and sodium chloride infusions in 10 normal patients is summarized in Table I.

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The sodium lactate employed in this investigation was supplied by the Eli Lilly & Company of Indianapolis, Ind.

TABLE I. COMPARISON OF EFFECTS OF MOLAR SOLUTIONS OF Na LACTATE AND Na CHLORIDE

PATIENT NO.		PLASMA Na (mEq./ml.)	GFR (ml./min.)	FILTERED LOAD Na (mEq./min.)	URINE FLOW (ml./min.)	URINE Na CONCENTRATION (mEq/ml.)	EXCRETION Na (mEq./min.)	PER CENT EXCRETION FILTERED Na	PER CENT EXCRETION FILTERED H_2O	PLASMA K (mEq./ml.)	EXCRETION K (mEq./min.)	PLASMA TOTAL SOLUTES (mOsm/L.)	URINE TOTAL SOLUTES (mOsm/L.)
Molar Sodium Lactate Infusion.—													
1	C	0.1430	114.0	15.80	4.3	0.1778	0.77	4.92	3.9	0.00441	0.0689	267	390
	E	0.1436	113.0	16.23	10.5	0.1850	1.91	11.47	9.3	0.00430	0.1654	284	424
2	C	0.1495	113.5	16.91	7.2	0.1788	1.29	7.63	6.3	0.00454	0.0701	297	421
	E	0.1511	146.3	22.17	16.4	0.1782	2.90	13.08	11.2	0.00441	0.1311	314	386
3	C	0.1411	108.0	15.25	3.3	0.1980	0.65	4.0	3.1	0.00404	0.0996	290	594
	E	0.1413	140.0	19.78	7.5	0.2070	1.55	7.8	5.4	0.00407	0.2336	286	569
4	C	0.1383	105.0	14.58	13.9	0.0278	0.386	2.6	13.0	0.00584	0.0644	293	95
	E	0.1697	92.0	15.63	16.2	0.0479	0.776	5.0	17.6	0.00498	0.0968	337	135
5	C	0.1436	229.0	32.58	4.1	0.1742	0.71	2.2	1.8	0.00474	0.1599	295	509
	E	0.1515	257.0	30.88	4.9	0.1948	0.95	3.1	1.9	0.00441	0.1890	303	530
6	C	0.1450	126.0	18.36	2.8	0.2470	0.69	3.8	2.2	0.00442	0.0678	288	280
	E	0.1501	13.1	19.63	4.6	0.2601	1.20	6.1	3.5	0.00424	0.0822	306	237
* Means	C	0.1434	133.0	18.91	5.9	0.1673	0.99	5.2	4.4	0.00467	0.0885	288	381
	E	0.1512	147.0	20.72	10.0	0.1788	1.79	8.6	6.8	0.00440	0.1497	305	380
Molar Sodium Chloride Infusion.—													
7	C	0.1420	141.0	20.00	5.2	0.1538	0.80	4.0	3.7	0.00443	0.0952	298	423
	E	0.1467	151.0	22.05	5.8	0.1872	1.09	4.9	3.8	0.00405	0.1084	306	452
8	C	0.1352	86.0	11.67	2.0	0.2170	0.43	3.7	2.3	0.00484	0.0373	289	535
	E	0.1472	88.0	13.44	4.0	0.2770	1.10	8.2	4.5	0.00548	0.0544	296	456
9	C	0.1540	100.0	15.47	2.4	0.1737	0.42	2.7	2.4	0.00507	0.0446	436	302
	E	0.1382	112.0	15.34	3.0	0.2490	0.75	4.9	2.7	0.00499	0.0681	306	547
10	C	0.1377	108.0	14.90	5.1	0.2163	1.10	7.4	4.7	0.00481	0.1014	290	472
	E	0.1448	113.0	16.35	6.2	0.2047	1.27	7.8	5.5	0.00471	0.1221	296	479
* Means	C	0.1422	109.0	15.51	3.7	0.1902	0.70	4.5	3.4	0.00479	0.0696	328	433
	E	0.1442	116.0	16.81	4.8	0.2295	1.10	6.5	4.1	0.00481	0.0825	301	484

C = Control.
E = Experimental.
*Mean of three 10 minute control periods and three 10 minute experimental periods.

After infusion of one molar sodium lactate there was an increase in the plasma sodium and total solute concentrations, filtered sodium load, urine flow, and sodium excretion per minute (Figs. 1, 2, and 3). The percentage excretion of the filtered sodium and filtered water significantly increased in equal increments. Hence, the urine sodium concentration and total urinary solute concentration remained at control levels. There was a tendency for the glomerular filtration rate to increase in all 3 experimental periods.

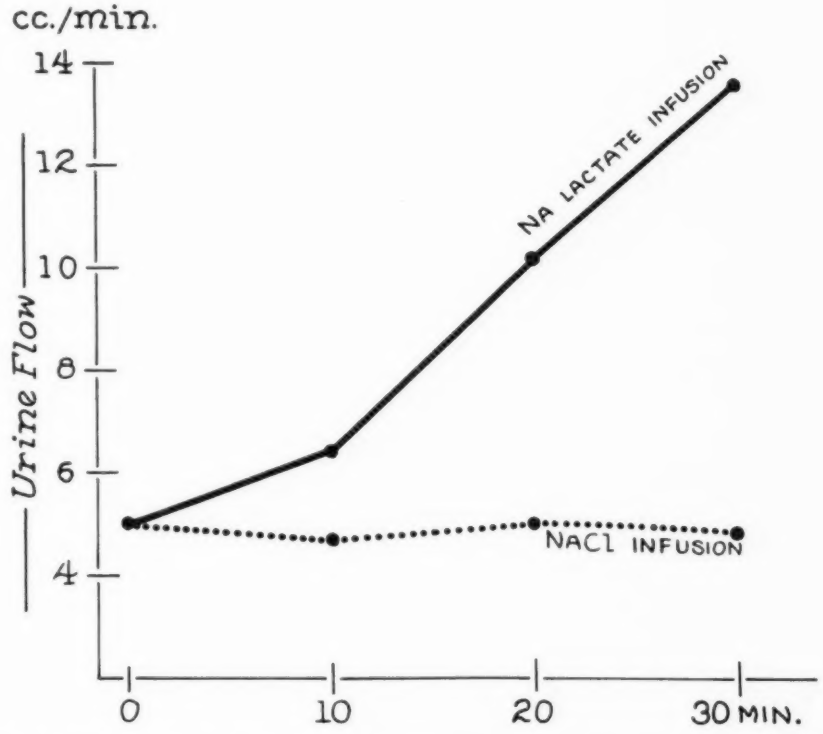

Fig. 1.—Comparison of mean urine flow per minute after infusion of molar sodium lactate and sodium chloride.

In comparison to the infusion of one molar sodium lactate, one molar sodium chloride showed no significant increase in serum sodium concentration. The glomerular filtration rate, filtered sodium load, and urine flow remained close to control levels. There was an increase in the sodium and total solute excretion per minute concomitant with an increase in the urine sodium and total solute concentration.

Infusion of sodium lactate caused a slight decrease in serum potassium concentration, with a concomitant increase in potassium excretion.

Sodium chloride had no effect on the serum potassium concentration or urinary potassium excretion.

There was 16.6 per cent excretion of the infused sodium after sodium lactate administration as opposed to 10.7 per cent excretion of the infused

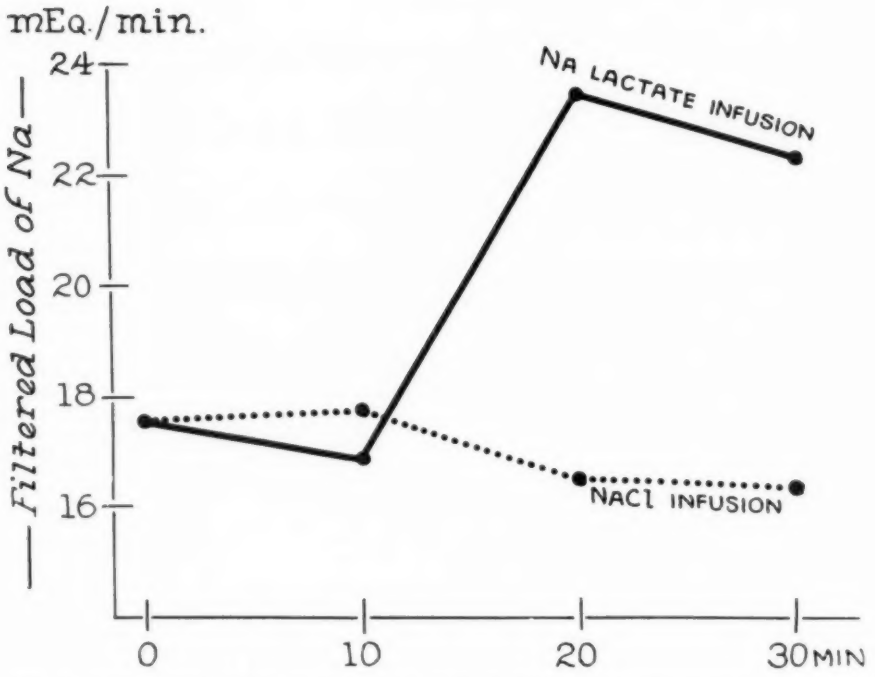

Fig. 2.—Comparison of the mean filtered load of sodium (milliequivalents per minute) after infusions of molar sodium lactate and sodium chloride.

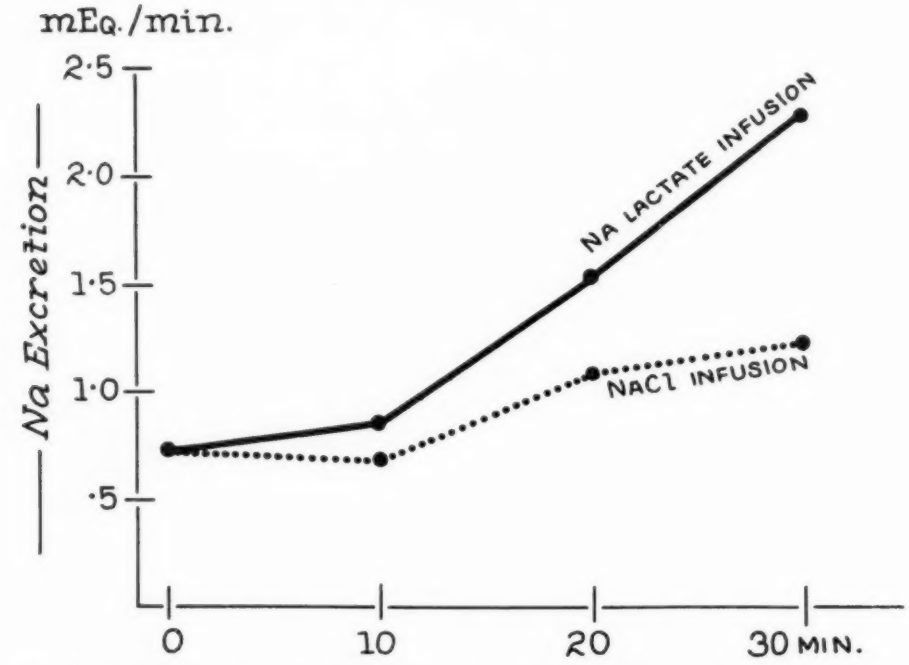

Fig. 3.—Comparison of mean sodium excretion per minute after molar sodium lactate and sodium chloride infusions.

sodium after sodium chloride injection. Furthermore, in the 30 minutes following 280 ml. sodium lactate infusion there was 301 ml. of urine excreted or 108 per cent of the volume infused. After sodium chloride infusion, 145 ml. of urine was excreted, or only 51 per cent of the infused volume (Table II).

DISCUSSION

From our results it is apparent that infusion of one molar sodium lactate (280 ml. in 30 minutes) enhances the renal excretion of sodium and water in normal patients (Tables I and II). The renal tubules are capable of responding in a constant uniform and sustained fashion to an incremental increase in sodium lactate infusion. The mechanism by which this is accomplished appears to be both an increase in the filtered sodium load and an increase in the percentage excretion of both sodium and water. One molar sodium chloride showed no increase in filtered sodium load or water excretion, 49 per cent of the infused volume being retained (Table II).

TABLE II. COMPARISON OF SODIUM AND WATER EXCRETION AFTER THE RAPID INFUSION OF 280 ML. OF MOLAR SOLUTIONS OF Na LACTATE AND Na CHLORIDE

	Sodium Excretion	
Sodium Lactate.—		
Sodium lactate infusion	280	mEq.
Sodium excretion for 3 10 min. experimental periods	46.6	mEq.
Per cent of sodium excreted	16.6	
Sodium Chloride.—		
Sodium chloride infusion	280	mEq.
Sodium excretion for 3 10 min. experimental periods	29.9	mEq.
Per cent of sodium excreted	10.7	
	Water Excretion	
Sodium Lactate.—		
Sodium lactate infusion	280	ml.
Urine volume for 3 10 min. experimental periods	301	ml.
Per cent of infused volume excreted	108	
Sodium Chloride.—		
Sodium chloride infusion	280	ml.
Urine volume for 3 10 min. experimental periods	145	ml.
Per cent of infused volume excreted	51	
Per cent of infused volume retained	49	

The glomerular filtration rate was not significantly increased either with sodium lactate or sodium chloride and is apparently not a factor in the excretion of sodium and water under these conditions.[12-14] The enhanced excretion of sodium and water with the infusion of molar sodium lactate is not entirely known. The failure of a sodium and water diuresis after the infusion of hypertonic sodium chloride apparently was not a result of increased osmotic pressure of the plasma since both sodium lactate and sodium chloride were of the same osmolar concentrations (1,960 mOsm. per liter). Selkurt[11] has shown that an increase in the filtered sodium load while sodium reabsorption remains constant causes an increase in sodium and water excretion. After infusion of sodium lactate as opposed to that of sodium chloride, there was an increase in the filtered sodium load from 18.91 to 20.72 mEq. per milliliter (Table I).

It has been shown[13, 19] that various transport systems may not be altered to the same extent by the loading solutes and an excess of sodium may be excreted because the reabsorptive system fails to reabsorb quantitatively the enhanced sodium load. Finally, several investigators[20, 21, 22] have demonstrated that sodium and water excretion varies widely depending on the anion of the loading solute. Saturation of the reabsorbing system for HCO_3^-, SO_4^{--}, $Fe(CN)_6^{---}$, and $S_2O_3^{--}$ is far lower than that for chloride. Hence, a relative increase in HCO_3^{--} excretion after sodium lactate infusion as opposed to chloride infusion would cause an early saturation of the HCO_3^- transport system and an enhanced excretion of sodium and water. Increased quantities of this unreabsorbed solute would limit water absorption in the proximal tubules and cause delivery of an increased volume of proximal fluid to the more distal portion of the tubules.

Although large volumes of fluids which contain sodium are potentially capable of overloading the circulatory system,[9] sodium administered as sodium lactate, which is excreted rather easily by the kidney, would appear to have certain advantages over solutions which contain chloride.

SUMMARY

1. A comparison was made of the manner in which the normal kidneys excrete equimolar solutions of sodium lactate and sodium chloride.

2. One molar sodium lactate as opposed to one molar sodium chloride results in an increase in serum sodium, filtered sodium load, urine volume, and percentage excretion of sodium and water. Serum potassium concentration is decreased with a concomitant increase in potassium excretion.

3. There was 16.6 per cent excretion of the infused sodium after molar sodium lactate administration as opposed to 10.7 per cent excretion of the infused sodium after sodium chloride injection.

4. There was a 108 per cent excretion of the infused volume after sodium lactate infusion, whereas only 51 per cent of the infused volume was excreted after molar sodium chloride injection.

REFERENCES

1. Bellet, S., Wasserman, F., and Brody, J. I.: Effect of Molar Sodium Lactate in Increasing Cardiac Rhythmicity, J. A. M. A. **160:** 1293, 1956.
2. Bellet, S., Wasserman, F., and Brody, J. I.: Treatment of Cardiac Arrest and Slow Ventricular Rates in Complete A-V Heart Block: Use of Molar and Half-Molar Sodium Lactate, Circulation **11:** 685, 1955.
3. Bellet, S., Wasserman, F., and Brody, J. I.: Further Observations on the Cardiovascular Effect of Sodium Lactate: Effect in Normal Subjects and in Various Arrhythmias, Am. J. M. Sc. **231:** 274, 1956.
4. Blakemore, W. S., Zinsser, H. F., Kirby, C. K., Bellet, S., and Johnson, J.: The Use of Molar Sodium Lactate in Certain Arrhythmias Complicating Intracardiac Surgery, Ann. Surg. **144:** 511, 1956.
5. Hartmann, A. F., Perley, A. M., Basman, J., Nelson, M. V., and Asher, C.: Further Observations on the Metabolism and the Clinical Uses of Sodium Lactate, J. Pediat. **13:** 692, 1938.
6. Hartmann, A. F.: Treatment of Severe Diabetic Acidosis. A Comparison of Methods With Particular Reference to the Use of Racemic Na Lactate, Arch. Int. Med. **56:** 413, 1935.
7. Neubauer, R. A., and Porter, R. R.: Some Observations on the Use of Hypertonic Sodium Lactate in Chronic Renal Diseases, Clin. Res. Proc. **1:** 58, 1953.

8. Neubauer, R. A.: Certain Observations on the Uptake of Base in Uremia and Acidosis Due to Advanced Renal Disease, Am. J. M. Sc. **228**: 656, 1954.
9. Whiteleather, J. E., and Gardner, J. E.: Acute Pulmonary Edema Resulting From Intravenous Saline, J. Tennessee M. A. **40**: 326, 1947.
10. Frank, M. N., and Dreifus, L. S.: Hormonal and Electrolyte Influences in Congestive Heart Failure, Am. J. M. Sc. **229**: 638, 1955.
11. Selkurt, E. E.: Sodium Excretion by the Mammalian Kidney, Physiol. Rev. **34**: 287, 1954.
12. Selkurt, E. E., and Post, R. S.: Renal Clearance of Sodium in the Dog: Effect of Increasing Sodium Load on Reabsorptive Mechanism, Am. J. Physiol. **162**: 639, 1950.
13. Levy, M. M., and Ankeney, J. L.: Influence of Glomerular Filtration Rate Upon Renal Tubular Reabsorption of Sodium, Proc. Soc. Exper. Biol. & Med. **79**: 491, 1952.
14. Green, D. M., and Farah, A.: Influence of Sodium Load on Sodium Excretion, Am. J. Physiol. **158**: 444, 1949.
15. Bellet, S., Wasserman, F., and Brody, J. I.: Molar Sodium Lactate: Its Effect in Complete A-V Heart Block and Cardiac Arrest Occurring During Stokes-Adams Seizures and in the Terminal State, New England J. Med. **253**: 891, 1955.
16. Bellet, S., and Wasserman, F.: The Effects of Molar Sodium Lactate in Reversing the Cardiotoxic Manifestations of Hyperpotassemia. Report of 12 Cases, A. M. A. Arch. Int. Med. **100**: 565, 1957.
17. Overman, R. R., and Davis, A. K.: Application of Flame Photometry to Sodium and Potassium Determinations in Biological Fluids, J. Biol. Chem. **168**: 641, 1947.
18. Dreifus, L. S., Frank, M. N., and Bellet, S.: Determination of Osmotic Pressure in Diabetes Insipidus, New England J. Med. **251**: 1091, 1954.
19. Goodyer, A. V. N., and Glenn, W. W. L.: Excretion of Solutes Injected Into the Renal Artery of the Dog, Am. J. Physiol. **168**: 66, 1952.
20. Pitts, R. F., Ayer, J. L., and Schiess, W. A.: The Renal Regulation of Acid-Base Balance in Man. The Reabsorption and Excretion of Bicarbonate, J. Clin. Invest. **28**: 35, 1949.
21. Sartorius, O. W., Roemmelt, J. C., and Pitts, R. F.: The Renal Regulation of Acid-Base Balance in Man. The Nature of the Renal Compensation in Ammonium Chloride Acidosis, J. Clin. Invest. **28**: 423, 1949.
22. Rice, L., Frieden, J., and Smith, M.: Tubular Action of Mercurial Diuretics, Am. J. Physiol. **175**: 47, 1953.

DIFFERENCE IN DAILY ALBUMIN SYNTHESIS BETWEEN NORMAL MEN AND WOMEN AS MEASURED WITH I^{131}-LABELED ALBUMIN

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SERUM albumin concentrations are altered in patients with a number of different diseases such as cancer, tuberculosis, nephrosis, hyperthyroidism, and cirrhosis. The subject has been extensively reviewed.[1-3] Although an alteration in serum albumin concentration may be the result of an increase or decrease in plasma volume, the subnormal serum albumin concentration has been shown to be the result of subnormal total body albumin stores in diseases as cirrhosis, nephrosis, hyperthyroidism, cancer, regional enteritis, and ulcerative colitis.[4-8] In addition, an increased albumin turnover has been found with subnormal serum albumin concentrations in patients with hyperthyroidism, nephrosis, regional enteritis, ulcerative colitis, and certain patients with idiopathic hypoalbuminemia, whereas decreased albumin turnover has been found in association with subnormal serum albumin concentrations in patients with cancer and cirrhosis.[4, 5, 7, 9] In the reports on albumin turnover, patients have been grouped according to disease states rather than according to age and sex distribution within a disease state. Sex differences in albumin concentration are not reported in standard textbooks[10-12]; in a recent report in which concentrations for serum albumin are reported separately by sex, there is no statistically significant difference between the 2 groups (Table I).[3] However, the group

TABLE I. SERUM ALBUMIN CONCENTRATIONS IN MEN AND WOMEN WITHOUT KNOWN DISEASE[3]

SUBJECT	NUMBER	TOTAL PROTEIN (GM. %)	% ALBUMIN
Men	163	7.3	68.9
Women	22	7.1	68.5
Total	185		
Average ± standard deviation			68.9 ± 4.2

reported on was composed of hospital patients discharged without a diagnosis of significant disease and may represent a select group.[3] When normal individuals were studied with respect to red cell or hemoglobin concentrations,[13] total red cell volume,[14] body water and body fat,[15] pulmonary volumes and capacities,[16] total body potassium,[15] or certain renal function parameters[17] significant differences between the sexes were found. In the studies reported here, serum albumin concentration, total circulating albumin, total exchangeable albumin,

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and albumin turnover were found to differ in a small group of normal young men and women, although considerable overlapping in values in each group occurred.

MATERIALS AND METHODS

The individuals studied volunteered as control subjects for medical studies (Table II). The individuals were 18 to 22 years of age except for Patient 01-32-68 who was 30 years of age. The average age of the individuals studied was 21.0 years; the average woman was 19.8 years of age and the average man was 21.5 years of age. All subjects were in good health with no chronic or recent acute diseases. Subjects were weighed daily before breakfast and maintained constant weight throughout the 1 month period of study. Total caloric and total protein intake were estimated daily by trained dietitians who offered the subjects relatively constant diets during the study period. Each individual studied had normal liver function as estimated by serum bilirubin and alkaline phosphatase levels as well as normal cephalin-cholesterol flocculation and thymol turbidity tests.

Two days prior to, and throughout, the period of study the subjects received 10 drops of Lugol's solution every 8 hours. One hundred microcuries of I^{131} albumin (10 to 50 mg. of albumin) diluted to 10 ml. in normal saline was delivered intravenously via a calibrated syringe. Four milliliter oxalated blood samples were removed without stasis at 12 and 24 minutes, 1, 2, 4, 8, 12, and 24 hours, and daily thereafter. Quantitative urine collections were obtained daily for the entire period of study. Occasional creatinine determinations on 24 hour urines were obtained as a check on the constancy of the 24 hour urinary collections. Twice weekly thyroid and thigh in vivo radioactivity determinations confirmed the absence of selective iodide I^{131} capture by the thyroid.

One milliliter blood samples were counted in 4 ml. screw cap vials in a thallium activated sodium iodide well scintillation counter to a standard deviation of 2 per cent. One hundred milliliter aliquots of the 24 hour urine collections were counted in an 8 ounce screw capped bottle on the top of the well with the lead pig removed. Appropriate standards were counted each time the subjects' blood or urine radioactivities were measured. Two sets of standards were used corresponding to the serum (1 ml.) and urine (100 ml.) unknowns. The actual standards were dilutions of sodium iodide I^{131} which had been compared to appropriate dilutions of the original I^{131} albumin solutions made on the first day of each study. I^{131} albumin standards were not used throughout the 1 month study period since the albumin settled in the counting vials or jars and adhered to the glass walls, making reproducible counts impossible. Plotting the log of the counting rate of an I^{131} albumin standard against time, therefore, did not give the characteristic physical decay curve of I^{131}, whereas plotting the log of the counting rate of a sodium iodide I^{131} standard did give the proper decay curve.

For each subject 4 curves were plotted, of which 3 are shown in Fig. 1. The per cent of the I^{131} albumin which remained in the body was obtained by subtracting the daily per cent of the original I^{131} albumin dose which was excreted in the urine from 100 per cent. Stool radioactivity averaged less than 1 per cent of urine radioactivity on any day and was neglected in this calculation as was the amount of radioactivity in each serum sample. Not shown in Fig. 1 is the plot of the fraction of the injected dose present in 1 ml. of plasma. The per cent of the I^{131} albumin dose which remained intravascularly was obtained by multiplying the radioactivity in 1 ml. of plasma by the plasma volume and expressing this as a percentage of the total radioactivity which remained in the body. This is shown in the middle section in Fig. 1. From albumin concentration, the plasma volume, and the fraction of the total dose which is intravascular, total exchangeable albumin (TEA) may be calculated as

$$\frac{\text{plasma volume} \times \text{grams albumin per milliliter of plasma} \times 100}{\text{per cent IHSA* intravascular at equilibrium}} = \text{TEA}$$

Another variation of TEA calculation is the isotope dilution method: dividing the fraction of the IHSA per milliliter of plasma into the albumin concentration per milliliter

*IHSA = I^{131} human serum albumin.

TABLE II. CLINICAL DATA AND RESULTS OF IHSA DEGRADATION STUDIES

HOSPITAL NUMBER	AGE	SEX	WEIGHT (KG.)	DATE OF STUDY	ALBUMIN CONCENTRATION (GM. %)	PLASMA VOLUME (ML./KG.)	TCA* (GM./KG.)	TEA† (GM./KG.)	TCA × 100 % / TEA	% IHSA DEGRADED/ DAY	ALBUMIN DEGRADED (GM./KG./ DAY)	DAILY CALORIC INTAKE	DAILY PROTEIN INTAKE (GM.)
01-16-90	18	F	72.5	11/55	3.8	35.5	1.35	3.16	42.3	5.1	0.168	2,500	75
01-16-89	18	F	66	11/55	4.1	33.6	1.38	3.67	37.8	5.0	0.183	2,200	80
01-16-92	20	F	65	11/55	3.8	35.8	1.36	3.54	38.1	4.3	0.159	2,200	80
01-32-71	21	F	61.1	7/56	3.4	46.5	1.49	4.12	36.2	4.7	0.194		
01-32-70	22	F	65	7/56	3.8	37.0	1.29	3.77	34.3	4.7	0.177	2,000	70
01-32-68	21	F	76	7/56	3.85	39.5	1.22	4.20	29.0	4.2	0.177	2,500	90
01-32-65	20	F	65.5	7/56	3.9	39.0	1.52	4.25	35.8	5.2	0.221	2,000	75
01-32-67	21	F	54	7/56	3.6	44.5	1.60	4.45	36.0	4.1	0.182	1,900	75
01-32-62	20	F	55.5	7/56	3.7	---	---	4.76	---	4.5	0.214	2,000	70
01-32-61	19	F	83.3	7/56	3.6	32.4	1.17	3.46	33.8	5.0	0.173	1,200	55
01-86-52	20	F	59.5	8/57	3.6	42.3	1.52	3.78	40.2	4.3	0.163	3,400	125
01-86-53	18	F	55	8/57	3.7	40.0	1.48	3.70	40.0	4.1	0.150	2,875	125
	19.8		64.9		3.7	38.7	1.40	3.91	36.7	4.6	0.180	2,250	
01-16-87	21	M	75	11/55	4.1	44.0	1.80	4.28	42.0	6.4	0.274	3,500	140
01-16-88	21	M	74	11/55	4.4	36.6	1.61	4.41	36.4	5.2	0.230	3,200	125
01-22-18	18	M	70	2/56	4.0	32.8	1.31	3.83	34.0	5.6	0.215	2,400	100
01-21-95	18	M	73.5	2/56	4.0	39.7	1.59	4.80	33.3	5.0	0.236	3,000	110
01-15-86	21	M	82	2/56	4.0	39.0	1.56	4.82	32.4	5.0	0.240	2,100	80
01-23-20	21	M	85.0	2/56	4.0	46.3	1.83	4.60	39.8	6.3	0.290		
01-14-98	22	M	58.5	7/56	3.6	54.0	1.94	4.55	42.7	4.0	0.182	2,600	80
01-32-68	30	M	64.5	7/56	4.1	49.6	1.99	5.10	39.0	4.3	0.219	2,500	90
Average	21.5		72.8		4.0	42.8	1.70	4.55	37.5	5.2	0.236		
Over-all	21.0		68.0		3.85	40.4	1.58	4.16	37.0	4.85	0.202		

*TCA = total circulating albumin.
†TEA = total exchangeable albumin.

of plasma at distribution equilibrium as determined by the appearance of the horizontal asymptote in the middle portion of Fig. 1. Finally, the daily degradation rate was determined for each day of the study by dividing the amount of radioactivity excreted in the urine on that day by the amount of radioactivity still remaining in the body on the mid-point of that day. In the lower portion of Fig. 1 is plotted for each individual day the per cent of I^{131} albumin degraded on that date. The half-time obtained from the upper portion of the figure divided into the natural logarithm of one half, 0.693, should give an internal check on the per cent of iodinated albumin degraded each day. The slope of the per cent of IHSA which remained in the body (shown in the upper portion of the figure) is identical in all instances with the slope of the terminal portion of the plasma radioactivity curve when this is plotted for the entire period of study.

TABLE III. REPORTED DATA ON ALBUMIN TURNOVER

AUTHOR	TECHNIQUE USED	NUMBER STUDIED	$T_{1/2}$ DAYS	"ALBUMIN SYNTHESIS" GM./KG./DAY
London[27]	Oral N^{15} glycine	1	20	Not available
Sterling[4]	I^{131} albumin		14.0	Not available
Berson and co-workers[5]	I^{131} albumin	10	17.8	0.205
Armstrong and associates[28]	Oral S^{35} yeast	1	30.	Not available
	IV S^{35} plasma	1	25.	Not available
Volwiler and collaborators[18]	Oral S^{35} cystine	7	27.4	Not available
	IV S^{35} plasma	3	22.0	Not available
Bauman[29]	I^{131} albumin	9	14.0	0.22
Masouredis and Beeckmans[23]	IV glycine-2-C^{14}	2	33	Not available
	I^{131} albumin	2	12.4	Not available
Margen and Tarver[30]	IV S^{35} plasma or albumin	8	26.5	(0.18)
	I^{131} albumin	8	18.0	(0.41)

TABLE IV. COMPARISON OF DATA ON BODY CONTENT AND TURNOVER OF ALBUMIN BY SEX

	WOMEN			MEN		
ALBUMIN	AVERAGE	STANDARD DEVIATION	STANDARD ERROR OF MEAN	AVERAGE	STANDARD DEVIATION	STANDARD ERROR OF MEAN
Albumin concentration (Gm./100 ml.)	3.73	0.24	0.069	4.02	0.28	0.1
Plasma volume (ml./Kg.)	38.73	4.54	1.37	42.75	6.59	2.33
Total circulating albumin (Gm./Kg.)	1.40	0.10	0.03	1.70	0.24	0.1
Total exchangeable albumin (Gm./Kg.)	3.90	0.36	0.1	4.55	0.34	0.12
Per cent IHSA degraded/day	4.60	0.38	0.1	5.22	0.84	0.3
Albumin degraded (Gm./Kg./day)	0.180	0.02	0.01	0.236	0.03	0.01

RESULTS

The results are listed in Table II. The average albumin concentration for the 12 women was 3.7 Gm. per cent whereas the average albumin concentration in the 8 men was 4.0 Gm. per cent. The average plasma volume in the women was 38.7 ml. per kilogram, whereas the average plasma volume in the men was 42.8 ml. per kilogram. The total circulating albumin expressed in grams per kilogram of body weight was 1.4 for the women and 1.7 for the men.

The total exchangeable albumin was 3.91 Gm. per kilogram for the women and 4.55 for the men. The ratio of the total circulating albumin to the total exchangeable albumin was 36.7 per cent in the women and 37 per cent in the men. The per cent degraded per day was 4.6 for the women and 5.22 for the men with albumin turnover being 0.18 Gm. per kilogram per day for the women and 0.24 Gm. per kilogram per day for the men. The average caloric intake

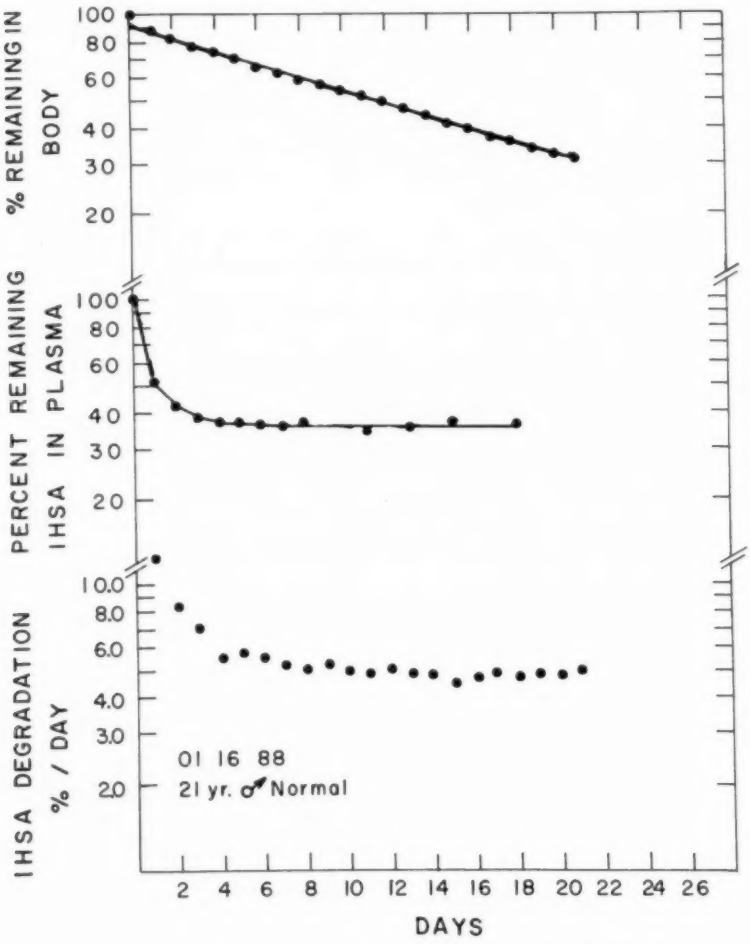

Fig. 1.—Representative data obtained during 1 month study of I^{131} albumin degradation. For interpretation see text.

for the women was 2,250 calories, whereas for the men it averaged 2,760 calories. The women ingested an average of 84 Gm. of protein per day, whereas the men ingested an average of 104 Gm. of protein per day. Unfortunately, basal metabolic rates and total body water determinations were not obtained on this group of patients so that comparison of albumin metabolism on the basis of active metabolizing body tissue, exclusive of fat, could not be made.

DISCUSSION

In previous investigations of I^{131} albumin metabolism and albumin concentration, men have been the subjects of study for the most part. The data presented here indicate that the man has a greater rate of albumin synthesis and breakdown than has the woman but this may be simply a reflection of his relatively greater active muscle mass or body water.[14, 15] Because of the availability of the recent comprehensive review of Volwiler and associates[18] of albumin metabolism, the data presented in Table III list only the recent estimates of albumin synthesis in adult individuals; the technique used for determination of albumin turnover is given.

The use of I^{131} albumin as an adequate tag for serum albumin has been seriously challenged.[5, 19-21] For comparative purposes and with carefully prepared I^{131} albumins, however, reproducible results may be obtained.[3, 20, 21] I^{131} albumin may not be an ideal tracer for body albumin since its behavior on DEAE cellulose is different from that of native serum albumin, although electrophoretically it may have the same mobility as serum albumin.[22] Criticisms have also been leveled against the use of S^{35} and C^{14} albumin turnover studies on the basis of reutilization of isotope.[18, 21, 23] It is not possible, therefore, to compare iodoalbumin data with data obtained when other labels were used since the S^{35}- or C^{14}-endogenously-, or even exogenously, labeled proteins are reutilized in the synthesis of albumin when the original molecule is broken down.

Further, the technique used for estimation of total exchangeable albumin varied markedly from author to author; widely varying estimates of total body albumin may be obtained from the same set of data depending upon whether the plasma curve is extrapolated to zero time or whether a kinetic analysis is used—assuming a certain pattern of body albumin distribution and degradation.[24, 25] This subject has been extensively reviewed recently.[21, 24-26] For this reason the data in Table III are not complete and data on albumin turnover in grams per kilogram per day are bracketed in those instances where an extrapolation method for calculation of total body albumin was used. Under these circumstances an erroneously high estimate of body albumin is obtained.[5, 21, 24]

In the present investigation, use of similar I^{131} preparations in normal men and women who were not convalescing from a chronic disease yielded absolute values for albumin turnover which differed significantly in several of the parameters related to body albumin stores and synthesis (Table IV). Sex differences in total circulating albumin, total exchangeable albumin, and albumin degradation in grams per kilogram per day are significant at $p=0.01$, whereas the sex differences in albumin concentration and daily per cent IHSA degradation are significant at the $p=0.05$ level for this small group of subjects.

SUMMARY

1. In 12 normal women the average albumin concentration was 3.7 Gm. per cent; the average circulating albumin was 1.4 Gm. per kilogram; the average total body albumin was 3.91 Gm. per kilogram; and the average rate of synthesis and degradation was 0.18 Gm. per kilogram per day.

2. In 8 normal men average serum albumin concentration was 4 Gm. per cent; the average total circulating albumin was 1.7 Gm. per kilogram; the average total body albumin was 4.55 Gm. per kilogram; and the average rate of synthesis and degradation was 0.24 Gm. per kilogram per day.

3. There is a difference in the rate of albumin synthesis and degradation between normal men and women which may be related to caloric intake, muscular activity, active metabolizing body tissue, or other as yet undetermined factors.

REFERENCES

1. Seibert, F. B., Seibert, M. V., Atro, A. J., and Campbell, H. W.: Variation in Protein and Polysaccaride Content of Sera in the Chronic Diseases, Tuberculosis, Sarcoidosis and Carcinoma, J. Clin. Invest. **26**: 90, 1947.
2. Gutman, A. B.: Plasma Proteins in Disease, Advances in Protein Chem. 4, New York, 1948, Academic Press, Inc.
3. Jencks, W. P., Smith, E. R. B., and Durrum, E. L.: The Clinical Significance of the Analysis of Serum Protein Distribution by Filter Paper Electrophoresis, Am. J. Med. **21**: 387, 1956.
4. Sterling, K.: Turnover Rate of Serum Albumin in Man as Measured by I^{131} Tagged Albumin, J. Clin. Invest. **30**: 1228, 1951.
5. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J.: Tracer Experiments with I^{131} Labeled Human Serum Albumin: Distribution and Degradation Studies, J. Clin. Invest. **32**: 746, 1953.
6. Blahd, W. H., Fields, M., and Goldman, R.: The Turnover Rate of Serum Albumin in the Nephrotic Syndrome as Determined by I^{131} Labeled Albumin, J. Lab. & Clin. Med. **46**: 747, 1955.
7. Gitlin, D., Janeway, C. A., and Farr, L. E.: Studies on the Metabolism of Plasma Proteins in the Nephrotic Syndrome. Albumin and Gamma Globulin and Iron-Binding Globulin, J. Clin. Invest. **35**: 44, 1956.
8. Steinfeld, J. L.: Hypoalbuminemia in Cancer Patients: Measurement of Total Circulating Albumin, Total Exchangeable Albumin, and Albumin Degradation Using I^{131} Albumin, Proceedings of the American Association for Cancer Research **2**: 149, 1956.
9. Steinfeld, J. L.: Hypoalbuminemia. Its Pathogenesis in Different Disease States, Soc. Nuclear Med., Oklahoma City Meeting, 1957.
10. Albritton, E. C., editor: Standard Values in Nutrition and Metabolism, Philadelphia, 1954, W. B. Saunders Company.
11. Sunderman, F. W., and Boerner, F.: Normal Values in Clinical Medicine, Philadelphia, 1949, W. B. Saunders Company.
12. Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry, ed. 13, New York, 1954, The Blakiston Company.
13. Wintrobe, M. M.: Clinical Hematology, ed. 3, Philadelphia, 1951, Lea & Febiger.
14. Huff, R. L., and Feller, D. D.: Relation of Circulating Red Cell Volume to Body Density and Obesity, J. Clin. Invest. **35**: 1, 1956.
15. Moore, F. D., McMurrey, J. D., Parker, H. V., and Magnus, I. C.: Symposium: Water and Electrolytes; Body Composition; Total Body Water and Electrolytes; Intravascular and Extravascular Phase Volumes, Metabolism **5**: 447, 1956.
16. Comroe, J. H., Jr., Forster, R. E., II, Dubois, A. B., Briscoe, W. A., and Carlsen, E.: The Lung: Clinical Physiology and Pulmonary Function Tests, Chicago, 1955, Year Book Publishers, Inc.
17. Smith, H. W.: Principles of Renal Physiology, New York, 1956, Oxford University Press.
18. Volwiler, W., Goldsworthy, P. D., MacMartin, M. P., Wood, P. A., Mackay, I. R., and Fremont-Smith, K.: Biosynthetic Determination With Radioactive Sulfur of Turn-Over Rates of Various Plasma Proteins in Normal and Cirrhotic Man, J. Clin. Invest. **34**: 1126, 1955.
19. Luck, J. M.: On the "Turnover" of Plasma Proteins, Am. J. Med. **20**: 317, 1956.
20. Steinfeld, J. L., Greene, F. E., Tabern, D. L., Paton, R. R., and Flick, A. L.: Degradation of Iodinated Human Serum Albumin Prepared by Various Procedures, J. Lab. & Clin. Med. **51**: 756, 1958.
21. McFarlane, A. S.: Use of Labelled Plasma Proteins in the Study of Nutritional Problems, Progr. Biophys. **7**: 115, 1957.
22. Fahey, J. L., and Steinfeld, J. L.: Chromatographic Differences Between Radioiodinated Albumin Preparations and Normal Human Serum Albumin, Proc. Soc. Exper. Biol. & Med. **97**: 281, 1958.

23. Masouredis, S. P., and Beeckmans, M. L.: Comparative Behaviour of I^{131} and C^{14} Labeled Albumin in Plasma of Man, Proc. Soc. Exper. Biol. & Med. **89**: 281, 1958.
24. Cornfield, J., Steinfeld, J. L., and Greenhouse, S.: Biomathematical Models for the Interpretation of Medical Experiments Using Isotopically Labelled Tracer Compounds, International Statistical Congress, Stockholm, Sweden, Autumn, 1957.
25. Lewallen, C. G., Berman, M., and Rall, J. E.: Studies of Iodoalbumin Metabolism. A Mathematical Approach to the Kinetics, J. Clin. Invest. **38**: 66, 1959.
26. Beeken, W. L., Volwiler, W., Goldsworthy, P. D., Wood, P. A., MacMartin, M. P., and Westort, C.: Comparative Analysis of I^{131} Albumin Metabolism and Distribution, Clin. Res. **7**: 114, 1959.
27. London, I. M.: Studies in the Rates of Formation of Serum Proteins in Man, in Youmans, J. B., editor: Plasma Proteins, Springfield, Ill., 1950, Charles C Thomas, vol. 2, pp. 72-82.
28. Armstrong, S. H., Jr., Bronsky, D., and Hershman, J.: The Persistence in the Blood of Radioactive Label of Albumins, Gamma Globulins and Globulins of Intermediate Mobility. Comparison of Die-away Plots Following Oral and Intravenous Administration of the S^{35} Label to the Same Subjects, J. Lab. & Clin. Med. **46**: 857, 1955.
29. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A.: Distribution and Metabolism of I^{131} Labeled Human Serum Albumin in Congestive Heart Failure With and Without Proteinuria, J. Clin. Invest. **34**: 1359, 1955.
30. Margen, S., and Tarver, H.: Comparative Studies on the Turnover of Serum Albumin in Normal Human Subjects, J. Clin. Invest. **35**: 1161, 1956.

SEPARATION OF 6.6S and 18S GAMMA GLOBULINS WITH ISOHEMAGGLUTININ ACTIVITY

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THE anti-A and anti-B isohemagglutinins of man are plasma proteins recognized by their ability to react with the appropriate erythrocyte substances, the A and B isohemagglutinogens, respectively. The isohemagglutinins are known to be gamma globulins.[1, 2] Immunologic studies indicated that the isohemagglutinins were heterogeneous,[3, 4] and recent immunochemical studies by McDuffie and co-workers[5] showed that isohemagglutinin activity was possessed by more than one type of protein molecule.

With the demonstration that the 18S and 6.6S gamma globulin groups could be separated by anion-exchange cellulose chromatography,[6] an investigation of the distribution of isohemagglutinin activity in the gamma globulin fractions was undertaken. Both the anti-A and anti-B isohemagglutinins were found in two distinct chromatographic gamma globulin fractions. Ultracentrifugal studies of the two fractions indicated that in one fraction the activity was associated with 18S gamma macroglobulins, in agreement with the observations of Pedersen[7] and Deutsch and co-workers.[1] In the other fraction isohemagglutinin activity was found to be a property of 6.6S gamma globulins.

MATERIALS AND METHODS

Sera were obtained from 2 normal men with group O erythrocytes. Neither had ever received blood transfusions, or blood group specific substance A or B injections. The isohemagglutinin titers were 1:256 for anti-A and 1:128 for anti-B in both sera.

Isohemagglutinin titers were determined on serial dilutions of serum or protein fractions in 0.9 per cent saline; a clean graduated serologic pipette was used for each dilution. One drop of diluted serum or protein fraction and 1 drop of a measured 2 per cent saline suspension of appropriate cells were delivered into 10 by 75 mm. tubes, incubated 30 minutes at 22° C., centrifuged 45 seconds at 900 g, and read macro- and microscopically. Reactions were graded according to Race and Sanger.[8] The titers given are a reciprocal of the highest dilution which showed at least a 1 plus reaction.

Zone electrophoretic fractionation of serum was carried out on polyvinyl chloride particle blocks and utilized about 1 ml. of serum.[6] After the protein content and isohemagglutinin activity in each block fraction were determined, the gamma globulin region was pooled. The gamma globulin pool and the whole serum from each subject were fractionated separately by chromatography on diethylaminoethyl cellulose[9] column. The procedure, described in detail elsewhere,[6] employed 150 ml. phosphate buffer, pH 8.0. The proteins were applied at low ionic strength (0.02M phosphate) and eluted by progressively increasing the ionic strength of the eluting solution. The effluent of the chromatogram

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was divided into 10 or more pools, concentrated by ultrafiltration to about 1 ml., dialyzed against buffered saline, pH 7.6 and tested for isohemagglutinin activity.

Chromatogram pools, collected in the regions between 5 to 15 per cent and between 60 to 85 per cent of the total elution volume, were fractionated by preparative ultracentrifugation techniques after adjustment of the effluent pool volume to 14 ml. and dialysis against buffered saline, pH 7.6. Ultracentrifugation was carried out as described by Robbins and associates[10] in a model L Spinco ultracentrifuge utilizing a No. 40 rotor at 39,000 r.p.m. for 150 minutes. Successive fractions were removed, starting at the top of the tube, and tested for activity.

RESULTS

Preparative electrophoretic fractionation of whole sera revealed the isohemagglutinin activity to be wholly within the gamma globulin region (Fig. 1). No difference was noted between the distribution of the anti-A and the anti-B activities.

Chromatographic fractionation of whole sera or of electrophoretically prepared gamma globulin revealed the isohemagglutinin activities to be eluted in 2 regions of the chromatogram (Fig. 2). The distribution of anti-A and anti-B activities was similar. A small amount of the isohemagglutinin activity was

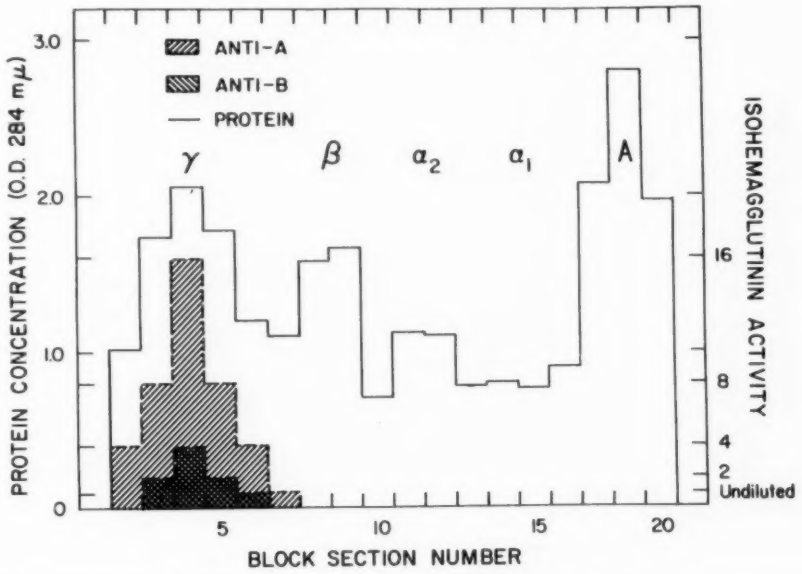

Fig. 1.—Isohemagglutinin distribution after serum fractionation by zone electrophoresis on a polyvinyl chloride particle block.

found in the region of 5 to 15 per cent of the effluent volume. In previous studies characterizing the proteins of this region only proteins with gamma globulin electrophoretic mobility and ultracentrifugal sedimentation coefficient of 6.6S were found in the 5 to 15 per cent effluent region.[6] This region has been termed gamma globulin fraction 1.

Most of the isohemagglutinin activity was eluted in the 60 to 85 per cent effluent region. The proteins of this region, gamma globulin fraction 5, are principally 18S gamma macroglobulins.[6]

Ultracentrifugal procedures were utilized to characterize further and to distinguish between the isohemagglutinins found in the 2 chromatogram fractions. Ultracentrifugation was carried out under conditions which cause only partial sedimentation of 6.6S gamma globulins but almost complete sedimentation of 18S gamma macroglobulins. The results of such a short term preparative ultracentrifugation are illustrated in Fig. 3. The activity in the 5 to 15 per cent

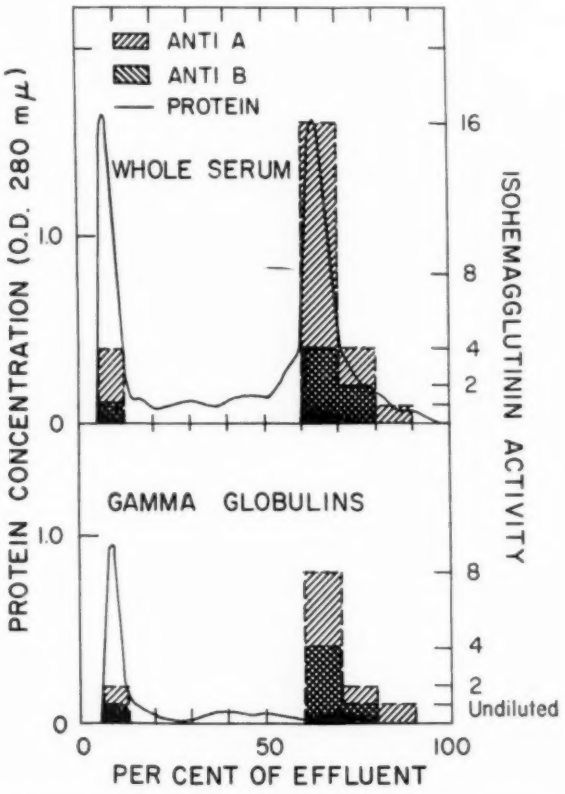

Fig. 2.—Isohemagglutinin distribution after anion-exchange cellulose chromatographic fractionation of whole serum and of gamma globulins obtained from whole serum by preparative electrophoresis.

chromatogram region sedimented slowly, and the activity in the 60 to 85 per cent region sedimented rapidly. The evidence clearly demonstrated that the 2 chromatographic isohemagglutinin fractions had different physicochemical properties. These activities apparently are associated with 6.6S gamma globulins in fraction 1 and 18S gamma macroglobulins in fraction 5.

DISCUSSION

The physicochemical demonstration of 2 different molecules which react in a similar manner in the test for isohemagglutinins confirms and extends the immunochemical evidence for 2 or more such components in human sera obtained

with anti-A:A substance and anti-B:B substance specific precipitates.[5] Similar distribution of activities between gamma globulin fractions 1 and 5 have been found for the Rh antibodies, anti-Rh$_0$ (D) and anti-rh′ (C) in hyperimmune sera[11] and of antiliver nucleoprotein activity in lupus erythematosus sera.[12] Many other antibody and antibody-like activities, however, were found only in fraction 1 or fraction 5.[11]

Abelson and Rawson[13] recently reported substituted cellulose chromatographic fractionation of serum isohemagglutinin activity into 3 fractions, one of which was identified as a 6.6S gamma globulin. Because whole serum was employed as a starting material in their chromatographic procedures, the other chromatogram fractions which have isohemagglutinin activity were composed of

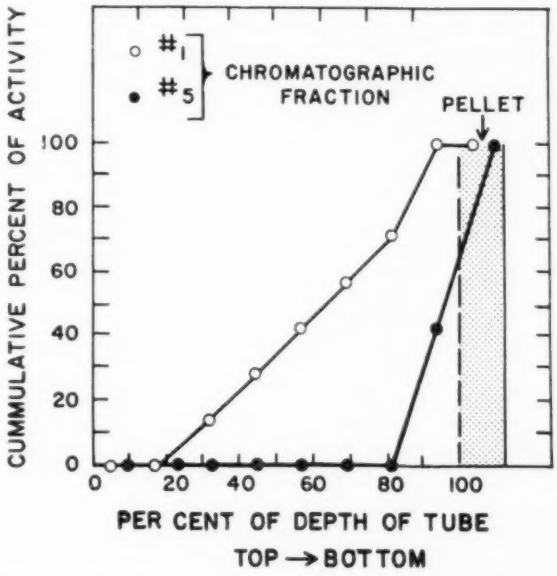

Fig. 3.—Ultracentrifugal sedimentation differences between the isohemagglutinin activities found in chromatographic gamma globulin fractions 1 and 5. Ultracentrifugation was conducted for 150 minutes at 39,000 r.p.m. at 5° C. Serial samples were withdrawn from the tube by means of a sampling pipette and tested for activity. Cumulative isohemagglutinin activity is plotted against the depth of the tube.

several protein components as shown by analytic electrophoresis and ultracentrifugation. The authors did not report which of these components possessed isohemagglutinin activity. They did find, however, that individuals with abnormal or unusual exposures to A or B blood group substances varied in the relative amount of activity in the several chromatogram fractions. It would be of interest to know what conditions increase the 6.6S isohemagglutinins and what circumstances increase the 18S isohemagglutinins. It would also be of interest to know whether these 2 isohemagglutinins react at the same antigen site on the red blood cell or at different sites. Anion-exchange cellulose chromatography as performed here would appear to be a relatively easy method of separating these activities for further study.

SUMMARY

The anti-A and anti-B isohemagglutinin activities have each been separated into 2 fractions by anion-exchange (DEAE-) cellulose chromatography. Both fractions have been identified as gamma globulins, one having a sedimentation coefficient of about 6.6S and the other of about 18S. In 2 normal blood group O donors the amount of activity in the 18S gamma globulin fraction exceeded the amount of activity in the 6.6S gamma globulins.

REFERENCES

1. Deutsch, H. F., Alberty, R. A., Gosting, L. J., and Williams, J. W.: Biophysical Studies of Blood Plasma Proteins, J. Immunol. **56**: 183, 1947.
2. Payne, R., and Deming, Q. B.: Electrophoretic Mobility in Paper of Isoagglutinins, J. Immunol. **73**: 81, 1954.
3. Wiener, A. S.: Origin of Naturally Occurring Hemagglutinins and Hemolysins: A Review, J. Immunol. **66**: 287, 1951.
4. Owen, R. D.: Heterogeneity of Antibodies to the Human Blood Groups in Normal and Immune Sera, J. Immunol. **73**: 29, 1954.
5. McDuffie, F. C., Kabat, E. A., Allen, P. Z., and Williams, C. A., Jr.: An Immunochemical Study of the Relationship of Human Blood Group Isoantibodies to γ_1 and γ_2 Globulins, J. Immunol. **81**: 48, 1958.
6. Fahey, J. L., and Horbett, A. P.: Human Gamma Globulin Fractionation on Anion-Exchange Cellulose Columns, J. Biol. Chem. **234**: 2645, 1959.
7. Pedersen, K. O.: Ultracentrifuge Studies on Serum and Serum Fractions, Uppsala, 1945, Almqvist & Wiksells Boktryckeri-A.-B.
8. Race, R. R., and Sanger, R.: Blood Groups in Man, ed. 2, Springfield, Ill., 1954, Charles C Thomas.
9. Peterson, E. A., and Sober, H. A.: Chromatography of Proteins. I. Cellulose Ion-Exchange Adsorbents, J. Am. Chem. Soc. **78**: 751, 1956.
10. Robbins, J., Petermann, M. L., and Rall, J. E.: Thyroglobulin in Serum After I^{131} Therapy. II. Sedimentation in the Ultracentrifuge, J. Biol. Chem. **208**: 387, 1954.
11. Fahey, J. L.: Separation of Serum Antibody Activities by Anion-Exchange Cellulose Chromatography. Science. In press.
12. Goodman, H. C., Fahey, J. L., Malmgren, R., and Brecher, G.: Separation of Factors in Lupus Erythematosus Serum Reacting With Components of Cell Nuclei, Lancet. **2**: 382, 1959.
13. Abelson, N. M., and Rawson, A. J.: Studies of Blood Group Antibodies, Fractionation of Anti-A and Anti-B Isohemagglutinins by Anion-Cation Cellulose Exchange Chromatography, J. Immunol. **82**: 435, 1959.

EXPERIENCE WITH PROLONGED ADMINISTRATION OF INTRAVENOUS FAT EMULSIONS

Behavior, Course, and Laboratory Findings in Dogs

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DESPITE continued experimentation following the early efforts of Yamakawa[1] to develop a fat emulsion for intravenous administration, a fat emulsion has yet to be formulated that may be administered safely in therapeutic quantities over a prolonged period of time. Since the high caloric content of lipids should make fat emulsions ideal as a continuing source of energy to patients, a study of the reactions to long-term administration of fat emulsions was undertaken.

Adverse reactions to fat emulsions may be divided into 2 categories: immediate and late. Immediate reactions encompass symptoms such as fever, chills, and nausea.[2, 3] The late reaction, sometimes referred to as the overloading syndrome, includes anemia,[4, 5] lipemia,[6, 7] gastrointestinal bleeding,[3, 4, 6, 8] impaired liver function,[9] and other abnormalities. These reactions need to be clearly delineated so that further investigation can be directed toward their elimination. To produce these late reactions, normal dogs were infused with 3 or 4 times the clinically recommended amount of fat emulsion. The effects of infusion of large quantities of 2 intravenous fat emulsions are presented with attention directed to the toxic changes which occurred.

METHODS

Dogs were selected after being maintained in the animal colony for a minimum of 2 weeks. Some dogs were then started on the infusions, whereas others were observed up to 2 more weeks to establish a base line for feeding habits and for changes in weight, hemoglobin, and white cell counts. Five control dogs that received no intravenous fluids were subjected to the same studies as the 21 experimental animals.

Under intravenous Nembutal anesthesia, a size 8 French polyvinyl (Pharmaseal) tube was inserted into the external jugular vein. The plastic tube was tunneled subcutaneously to the dorsum of the neck and exteriorized. The tube was connected to a Touhy-Borst adapter which was secured to a leather dog harness. The entire area was wrapped in canvas to prevent the dog from disturbing the apparatus. In the event the plastic tube came out of the vein, other cutdowns were performed on the opposite external jugular vein, on the femoral veins, on smaller vessels, or, eventually, only ordinary venipunctures were used.

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TABLE I. SOME OBSERVATIONS OF LIPOMUL I.V. AND SR TREATED DOGS

DOG NO.	WEIGHT (KG.) INITIAL	WEIGHT (KG.) LAST	EMULSION	NO. OF DAYS ON STUDY	OUTCOME	DAILY DOSE ML./KG.	DAILY DOSE GM./KG. FAT	NO. DOSES GIVEN	DOSE LIPEMIA NOTED	TOTAL ML. EMULSION GIVEN	DOSE-DAYS	HEMOGLOBIN FIRST	HEMOGLOBIN LAST
8019	16.0	16.2	Lipomul I.V.	44	Lived	30	4.5	30		14,400	900	12.7	8.5
8020	14.0	16.0	Lipomul I.V.	44	Lived	30	4.5	30		11,400	900	13.1	12.3
6066	17.0	16	Lipomul I.V.	11	Died	35.3	5.3	8	2	4,800	282	11.0	10.7
5125	16.0	12	Lipomul I.V.	54*	Died	37.5	5.6	28	1	16,625	1,050	19.3	3.5
4142	15	—	Lipomul I.V.	15	Died	40	6.0	11		6,600	440	12.0	7.2
5116	15.0	12	Lipomul I.V.	37†	Died	40	6.0	18	5	10,800	720	16.2	9.0
8101	10.0	9.0	Lipomul I.V.	26	Died	40	6.0	20	9	8,500	800	13.2	6.3
8103	11.2	9.6	Lipomul I.V.	16	Died	40	6.0	12	9	5,400	480	13.7	5.5
4118	12.5	10	Lipomul I.V.	25	Died	48	7.2	19	16	11,400	912	14.1	5.5
4175	12.0	—	Lipomul I.V.	11	Died	50	7.5	9	8	5,400	450	14.1	14.5
6092	11.0	8.8	Lipomul I.V.	11	Died	54.5	8.2	8		4,800	436	12.7	8.1
										Average:	670		
6105	16.0	16.3	SR	39	Lived	31.3	4.7	24		11,800	752	16.5	12.3
6163	16.3	16.7	SR	32	Lived	34.7	5.2	18		8,900	624	13.0	10.5
1540	9	12.6	SR	51	Lived	40	6.0	30		10,800	1,200	7.8	8.5
1544	13	13.4	SR	43	Lived	40	6.0	30		15,600	1,200	13.4	8.3
6188	16.4	16.0	SR	42	Lived	40	6.0	30		18,900	1,200	17.6	10.2
7026	19.0	16.3	SR	43	Lived	40	6.0	30		22,920	1,200	13.3	8.3
8179	12.0	10.6	SR	25	Died	40	6.0	17		8,160	680	14.1	6.9
8186	11.5	11.4	SR	62	Lived	40	6.0	30		13,800	1,200	12.2	7.2
7117	13.8	13.6	SR	18	Died	50	7.5	13	12	8,970	650	10.6	10.7
7138	18.2	16.0	SR	25	Died	50	7.5	18	13	15,370	900	11.9	8.5
										Average:	960.6		
6166	13.0	13.0	Control	73	Lived							15.7	14.5
7077	12.8	14.2	Control	63	Lived							12.3	16.2
8021	15.0	16.2	Control	56	Lived							15.3	16.3
8072	15.8	13.2	Control	36	Lived							13.7	12.0
8183	12.8	13.2	Control	31	Lived							14.1	13.4

*There was a 17 day interval between infusions 14 and 15.
†There was a 17 day interval between infusions 13 and 14.

The two intravenous fat emulsions used in these experiments were Lipomul I.V.* and an emulsion specially made for these studies, referred to as SR.† The two emulsions are composed of 15 per cent cottonseed oil and differ only in their emulsifier-stabilizer systems. Both preparations contain Pluronic F-68 (polyoxyethyleneпropylene) as a coemulsifier. Lipomul I.V. employs a phosphatide, lecithin, as the emulsifier. SR contains 1.25 per cent polyethylene glycol 400 (mono) stearate (a nonphosphatide emulsifier), with 0.5 per cent TEM 4T (acetylated tartaric acid ester of monoglycerides) as the stabilizer.[10]

Intravenous fat emulsions were administered 5 times weekly beginning the day after the cutdown. The average infusion ran 60 drops per minute and took about 3 hours. The animal was able to move about the cage at will during those infusions administered through the external jugular vein. At the end of each infusion the plastic tube was filled with 1 to 2 ml. of a solution containing 20 mg. heparin per 50 ml. of 50 per cent dextrose to prevent clotting in the tube between infusions.

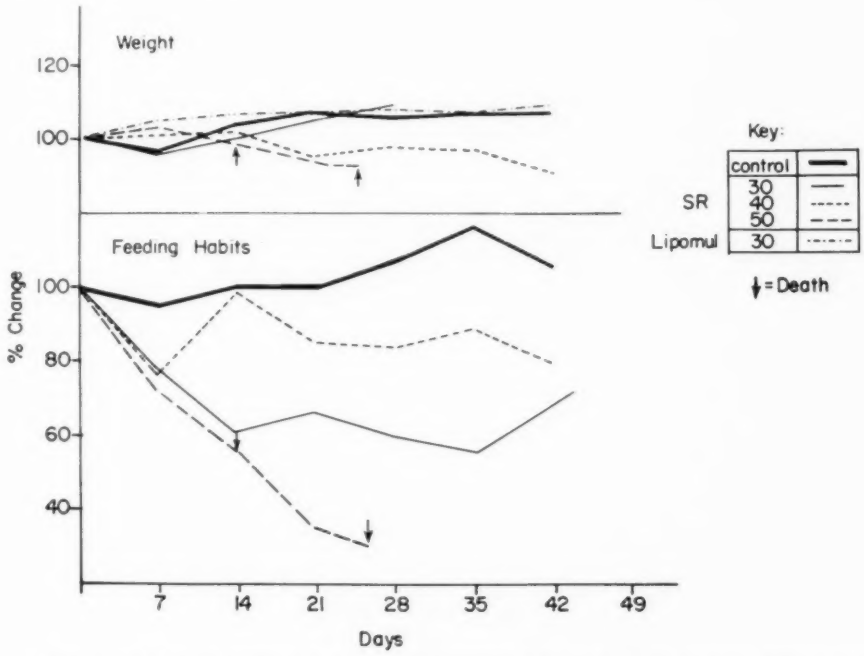

Fig. 1.—Effect of multiple infusions of Lipomul I.V. and SR at various dosage levels on change of weight and feeding habits in dogs. In this and subsequent charts each arrow represents the death of 1 dog.

The animals were fed Purina dog chow kibbled meal and water throughout the study. When feeding habits were followed, each day's food was weighed as was the uneaten portion which remained from the previous day. This served as an approximation of the general feeding habits of the animal, regardless of whether or not this may have been followed by regurgitation of the food.

Blood samples were drawn prior to the first and fifth infusion of each week. Complete blood counts, including platelet and reticulocyte counts, were done on all animals. Blood volume, serum protein, chlorides, potassium, sodium, blood urea nitrogen, and electrophoretic studies were obtained on some animals.

*All Lipomul I.V. used in this study was supplied by The Upjohn Company, Kalamazoo, Mich.

†The SR emulsion was made by the Southern Utilization Research and Development Division, United States Department of Agriculture, New Orleans, La.

The animals were observed daily during the period of fat administration. All animals except those retained for prolonged observation were subjected to complete autopsy. Duplicate sections were sent to the U. S. Army Medical Nutrition Laboratory* for a detailed study of the so-called intravenous fat pigment.

RESULTS

Clinical Findings.—The effects of daily infusions of varying amounts of intravenous fat emulsions are seen in Table I, and may be summarized by noting that animals survived the daily administration of 30 ml. per kilogram of Lipomul I.V., or 40 ml. per kilogram of SR, but did not survive when larger amounts of either emulsion were administered. The tolerance data were also evaluated by determining the average "dose-day" level of Lipomul I.V. and SR infused dogs (Table I). This was calculated by multiplying the dosage level for each dog by the number of infusions that the dog received. The average for Lipomul I.V. was 640 dose-days as compared to 960 dose-days for SR, a difference of 30 per cent.

The major clinical changes noted in these animals fell into 6 categories: (1) general activity, (2) weight, (3) feeding habits, (4) vomiting, (5) gastrointestinal bleeding, and (6) cough and dyspnea.

General activity: There was a significant decrease in general activity during the hours of intravenous fat administration; this was most prominent in those receiving the highest dosage level of both fats. The dogs usually rested quietly in their cages during the administration of the fat emulsion, and would often remain so for 2 or 3 hours after completion of the infusion. The animals were no longer frisky by the second or third week of infusion therapy.

Weight: The majority of animals receiving intravenous fat lost weight, regardless of which emulsion was used (Fig. 1, Table I). At dosage levels between 30 and 40 ml. per kilogram, weight losses ranged between 1 and 14 per cent for those receiving SR and between 6 and 25 per cent for those receiving Lipomul I.V.

Feeding habits: Oral intake decreased noticeably during the period of fat administration. Daily oral intake was measured for 6 animals receiving fat and for 3 control dogs. Daily oral intake was 72 to 78 per cent of normal by the end of the first week of fat administration (Fig. 1). In the group receiving 30 ml. per kilogram of SR, the intake fell to 43 per cent of normal during the last week of infusion. After the last infusion of fat, food intake rose to approximately 90 per cent of normal. Food intake in the group receiving 40 ml. per kilogram of SR fluctuated between 80 and 90 per cent of normal after the first week. Animals that died after receiving 50 ml. per kilogram of SR had lowered their food intake to 31 per cent of normal prior to death.

Vomiting: Vomiting occurred in over 80 per cent of all animals receiving both kinds of fat emulsion. The dogs that received Lipomul I.V. and did

*We wish to thank Major S. W. Thompson, U. S. Army Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colorado, for staining and evaluating the many tissues sent him from this project.

not survive first began to vomit with either the first or second infusion and continued vomiting intermittently until death. In no case was the vomiting of sufficient magnitude to cause death by fluid or electrolyte imbalance. The 2 dogs that survived the infusion of Lipomul I.V. at the rate of 30 ml. per kilogram stopped vomiting after the fifth infusion. Vomiting usually began during or shortly after the completion of the infusion and was of brief duration.

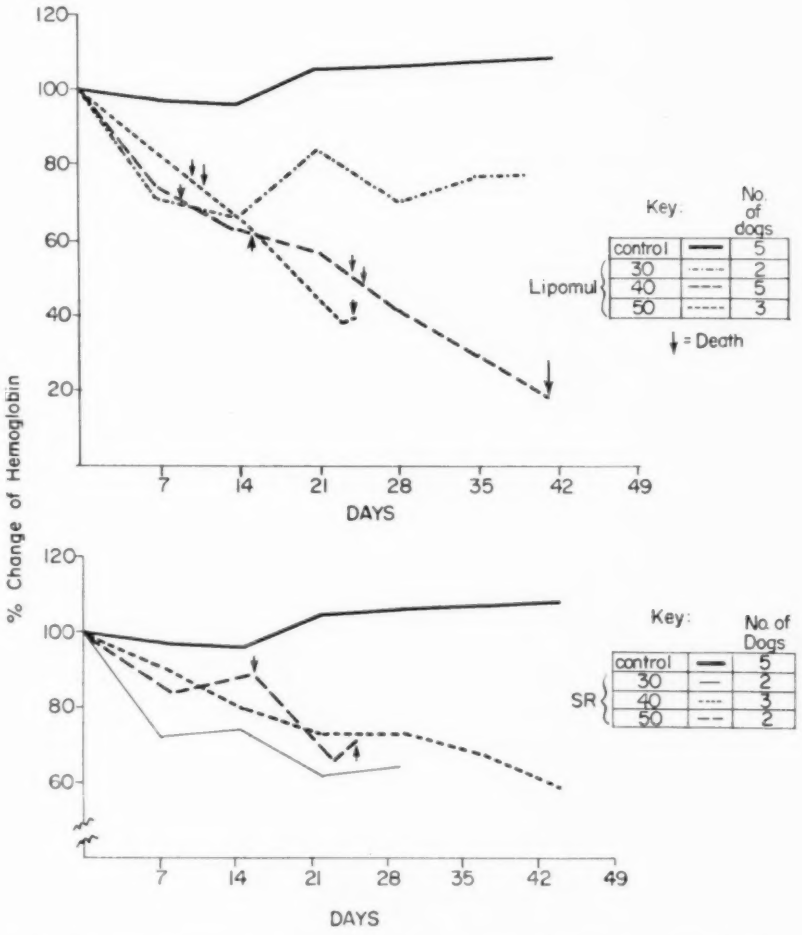

Fig. 2.—Per cent change in hemoglobin after multiple infusions of Lipomul I.V. and SR at 3 different dosage levels.

The vomitus was clear and watery except in the case of 1 dog which had hematemesis on the day of death. Animals that received SR did not vomit until the sixth or seventh infusion and usually stopped vomiting before the last week of infusions.

Gastrointestinal bleeding: Blood was grossly detectable in the stools of 4 dogs receiving Lipomul I.V. and 2 receiving SR. This was not seen until the last one or two infusions, and did not occur in the animals which survived

fat administration, except for 1 dog with an intussusception. In all 6 cases of melena, a gastrointestinal source of bleeding was found at autopsy; duodenal ulcers were found in 3, petechial hemorrhages in the duodenum or prepyloric area in 2, and an intussusception in 1.

Cough and dyspnea: Three animals that received Lipomul I.V. had a dry cough and dyspnea during the last few days of fat administration. Large pleural effusions were found at autopsy in each, and one had an apical lung abscess which had ruptured into the pleural cavity.

Lipemia: Nine of the 21 dogs (Table I) developed lipemia. All dogs with persistent lipemia died prior to completion of the originally proposed number of infusions. Of the 12 dogs that did not develop lipemia, 3 died. Lipemia frequently disappeared during the 2 day week end when fat emulsions were not given, but by the end of the succeeding week lipemia would again be present. Two dogs receiving Lipomul I.V. were able to clear the lipemia during a 2 week interruption of therapy, but when fat infusions were resumed lipemia reappeared after fewer infusions than in previously normal dogs. Of the 9 lipemic dogs, 7 were on Lipomul I.V. and 2 on SR. These results suggest that the overloading syndrome appears earlier in dogs receiving Lipomul I.V. than in those receiving SR.

Laboratory Studies.—The major emphasis in laboratory evaluation of these animals has been upon changes in the formed elements of the blood and upon the changes in serum proteins as measured by electrophoresis and salt fractionation. Serial studies revealed no significant changes in blood sugar, prothrombin time, Lee-White clotting time, direct and total bilirubin, or in platelet counts. There was a terminal increase in blood urea nitrogen in 4 animals, but all others remained within normal range. Bromsulphalein retention was within normal limits in 8 dogs, but 4 dogs receiving Lipomul I.V. and 1 dog receiving SR showed increases from 10 to 40 per cent retention.

Blood volume decreased from 12 to 13 per cent in the 3 dogs in which this was measured.

Hemoglobin: A normochromic normocytic anemia developed in all dogs which received intravenous fats (Fig. 2), but the anemia was more severe in those which received Lipomul I.V. than in those which received SR. There also appeared to be a correlation between the drop in hemoglobin and the dosage of intravenous fats given, but this did not become apparent until after the third week of therapy. A marked anemia was noted at the end of therapy in some animals.

Reticulocyte counts: Reticulocyte counts were an aid in the study of the progressive anemia. In this laboratory, the normal reticulocyte count of dogs ranges between 0.3 and 3 per cent. The reticulocyte count in the animals on intravenous fats did not indicate a marked response to the anemia but isolated elevations of 6 to 17 per cent were noted during or after infusions in 4 dogs.

White blood cell count: An elevation of the white blood cell count was observed during the first week of infusion. It was maintained throughout

the period of infusion, and was followed by a further precipitous rise just prior to death (Fig. 3).

Serum proteins: A detailed study of the changes in serum proteins of these dogs was performed by Singleton and co-workers[18] and is reported elsewhere.

Autopsy Findings.—

Survival experiments: Dogs given Lipomul I.V. at the rate of 30 ml. per kilogram or SR at the rate of either 30 or 40 ml. per kilogram survived 30 infusions, after which infusions were terminated. Dogs from each of these 3 programs were killed at the end of the experiment for an immediate evaluation of the changes following prolonged intravenous fat emulsion administration. Others were kept for long-term study. In 1 dog that received the higher dosage of SR fat, a significant finding was a 1.5 by 1.5 cm. ulcer (Fig. 4) on the anterior surface of the duodenum, 4 cm. distal to the pylorus.

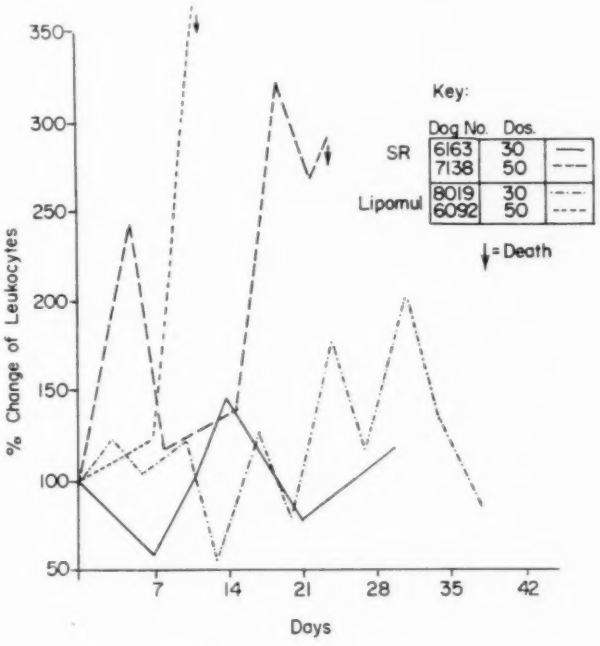

Fig. 3.—Per cent leukocyte change observed in dogs after multiple infusions of Lipomul I.V. and SR.

Another dog, No. 1544, had a 10 cm. intussusception of the terminal ileum. In none of these animals was there evidence of fatty degeneration of liver, spleen, or kidney, nor was there any unusual fatty deposition detected by special staining of the aorta.[3]

Histologic studies showed no significant abnormalities except for variable hyperplasia of the lymphoid tissues and bone marrow, and the presence of the intravenous fat pigment deposits in the liver, spleen, lymph nodes, and bone marrow.

Nonsurvival experiments: Nine dogs that received Lipomul I.V. at dosages higher than 30 ml. per kilogram died prior to completion of the planned program of therapy. The liver was generally light brown in color, greasy to the touch, and grossly enlarged. Eight had pneumonic changes and 6 had pleural effusions which were often bilateral.

Peritoneal fluid, present in 4 dogs, was usually lipemic, hemorrhagic, and scant in amount. The spleen was generally pale and contracted.

Abnormalities of the stomach or duodenum were found in 5 of the 9 dogs. These varied from classic peptic ulcers in 4 dogs (3 duodenal and 1 gastric) to a hemorrhagic mucosa in 1 dog.

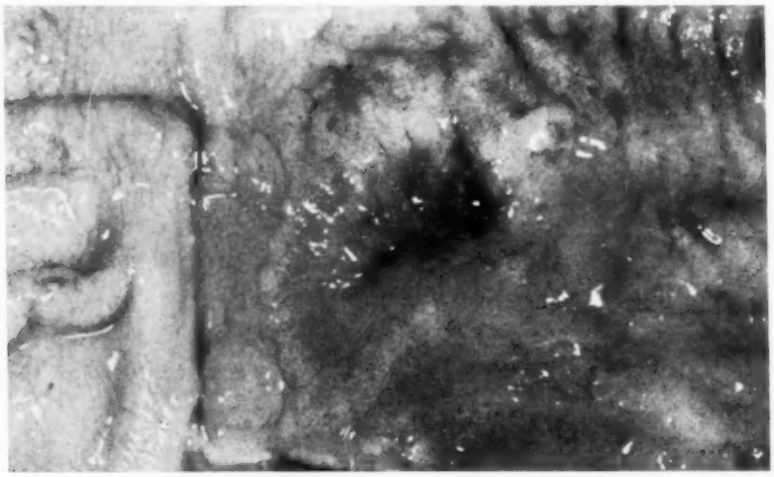

Fig. 4.—Duodenal ulcer in Dog 7026 killed after receiving 30 infusions of SR at a dose of 40 ml. per kilogram.

Two dogs that received SR at 50 ml. per kilogram did not survive. Both had grossly fatty livers, pneumonia, and pleural effusions. A large duodenal erosion was present in 1 dog in addition to areas of hemorrhagic mucosa in the upper small bowel. A large intra-abdominal abscess involving the tail of the pancreas, left adrenal, and left perirenal area was present in 1 animal that also had a serosanguinous pericardial effusion.

Histologic studies showed varying degrees of pneumonitis, atelectasis, congestion, and pulmonary edema in the majority, and septic emboli in 3 animals that died after infusion of either Lipomul I.V. or SR. In the livers of dogs which received Lipomul I.V. and SR were microgranulomata filled with pigment-laden histiocytes. The liver showed areas of focal necrosis in 4, varying amounts of fatty infiltration in 5, and congestion in 3 of the dogs receiving Lipomul I.V., and 2 of the dogs receiving SR had fatty infiltration. Fatty infiltration and degeneration of the renal tubules were noted in 2 dogs on Lipomul I.V. and 2 on SR.

The intravenous fat pigment was found in all animals which received Lipomul I.V. and SR in the cytoplasm of the reticuloendothelial system in the

liver, spleen, lymph nodes, and bone marrow. The intravenous fat pigment has not yet been identified in spite of extensive studies.[4]

Material for liver biopsies has been taken from 4 dogs which survived the infusion period. Dog 6188 received 18,900 ml. of SR over a period of 42 days. Three days after termination of infusions, the liver biopsy revealed aggregates of macrophages containing a foamy cytoplasm throughout the parenchyma of the liver. The intravenous fat pigment was identified within the cytoplasm of the macrophages. Four hundred days later a liver biopsy still revealed the intravenous fat pigment. In addition, aggregates of pigment-laden macrophages considered to be multinucleated giant cells were seen, especially at portal triad areas. Material for biopsy removed from 3 other dogs, Nos. 6163 (SR), 8020 (Lipomul I.V.), 8186 (SR), 300, 300, and 225 days after the last infusion also demonstrated the residual effect of the intravenous fat administration.

DISCUSSION

A safe and effective intravenous fat emulsion would be a valuable therapeutic aid to anyone unable to take adequate nutrition by the oral route. It is obvious from the indication for intravenous fat emulsions that their chief use would be in severely debilitated patients who were in need of nutritional supplements, fats being used because of their greater caloric value per gram than either proteins or carbohydrates. These thoughts prompted this study of some of the problems that might occur with the *protracted* use of intravenous fat emulsions.

Most of the immediate difficulties noted with some of the earlier emulsions (i.e., fever, back pain, chills, anxiety, flushing, nausea, and vomiting[2, 3]) are difficult to quantitate in the experimental animal, and in any case, seem to have been eliminated in the majority of patients with the more refined emulsions now available.

However, other critical toxic manifestations (i.e, anemia,[4, 5] hyperlipemia,[6, 7] serum protein alteration,[11, 12] and death[13]) seen in patients can be produced for study in the dog by the repeated administration of fat emulsions at levels 3 to 4 times that clinically recommended.

The development of anemia during fat therapy is a disturbing feature, and is a complication that could be ill-afforded by a patient with malnutrition. The decreased red cell count which follows successive infusions has been observed in patients and invariably develops in normal dogs infused with 4 to 6 grams of fat per days over long periods. Watkin[5] reported a fall in red cell count in 5 of 8 patients studied. Artz[4] reported a fall in hematocrit during the administration of fat emulsion in each of the 22 patients to whom he administered 10 or more infusions of Lipomul I.V. The drop in hematocrit may be due to hemolysis, erythropoietic depression, gastrointestinal bleeding, or any combination of these factors. Evidence for hemolysis was first studied by Loewy and co-workers[14] who measured increased biliary bilirubin excretion in dogs on a high fat diet. Later, Swank[15] noted erythrocytic spherocytosis during alimentary lipemia. Hartwig and Cohn[16] found hematocrit increases

up to 35 per cent after incubation of dog and human erythrocytes in fat emulsions. Another study implicating hemolysis as a possible factor in the anemia was conducted by Creditor[17] who found erythrocytes to be more sensitive to mechanical agitation in the presence of fat emulsion. One approach to the cause of hemolysis may lie in a study of the properties of the emulsifiers and stabilizers. The developmental rate of anemia was lower in dogs receiving SR fat emulsion than in those receiving Lipomul I.V. Further studies are being carried out with various emulsifiers to evaluate their role in hemolysis.

Changes in serum protein patterns also reflected variation in the influence of different emulsifiers and stabilizers on biologic systems. Singleton and associates[18] found a decrease in serum albumin of 35 to 40 per cent in dogs receiving Lipomul I.V. and 20 to 25 per cent in those animals infused with SR fat emulsion. Whenever serum albumin decreased to 40 per cent of its original value, as determined electrophoretically, death followed. The decrease in concentration of serum albumin was not due entirely to a corresponding drop in total serum protein, but was partially related to the formation of an albumin-lipid complex with a changed mobility. The formation of this complex appeared to be greater with Lipomul I.V. than with the SR emulsion.

Erythropoietic depression is suggested by the poor reticulocyte response which occurs in dogs which develop a normocytic, normochromic anemia.

Persistent lipemia has been recognized as an indication for discontinuation of fat emulsion and was found to develop in most of the dogs that eventually died during the infusion period. The serum in some of the dogs was so filled with fat that it was grossly indistinguishable from the fat emulsion which had been given the preceding day. Eventually the lipemia persisted for 72 hours during which no fats were administered. Once a persistent lipemia was noted the animals seemed to pursue a more rapid downhill course. Thus, lipemia in a patient on fat therapy should be taken as a sign to discontinue therapy rather than run the risk of producing the "overloading" syndrome. This seems all the more important in view of the absence of a persistent lipemia in all the animals that survived.

The development and persistence of histologic changes in the liver of the dog demonstrate a rapidly appearing and long lasting effect on the liver. Similar changes have been reported and studied in human tissue.[19] Liver biopsies performed as long as 400 days after the last intravenous fat infusion still revealed the presence of macrophages and the intravenous fat pigment. Impaired liver function has been reported after successive infusions of fat emulsions[9] but it is not known whether these changes are associated with macrophages and/or pigment deposition.

The presence of frank ulcers in the stomach or duodenum of one fourth of the animals in the study is interesting in view of the gastrointestinal bleeding in patients receiving intravenous fat.[3, 4, 6, 8] A study of the effect of intravenous fat emulsions on gastric secretions is now in progress.

The use of large quantities of intravenous fat provides a tool for the study in experimental animals of clinical reactions to intravenous fat emulsions

The importance of such a tool is underscored by the observation that many of the reactions previously observed in patients, such as anemia,[4, 5] vomiting,[3] lipemia,[6, 7] gastrointestinal bleeding,[3, 4, 6, 8] and even death,[13] have been reproduced in the experimental animal.

CONCLUSIONS

The effects of the chronic administration to normal dogs of 2 intravenous fat emulsions have been studied. The emulsions were administered in amounts 3 to 4 times those recommended clinically in order to study the toxic effects of intravenous fat emulsions.

The following reactions, previously reported in humans receiving intravenous fat emulsions, occurred in the experimental animals: lethargy, vomiting, persistent lipemia, anemia, gastrointestinal bleeding, abnormal liver function, and deposition of intravenous fat pigment.

Peptic ulcers were found in either the stomach or duodenum of 24 per cent of the dogs which received intravenous fat emulsions.

SR emulsion was administered to normal dogs without lethal results at a 40 ml. per kilogram dosage, whereas Lipomul I.V. was consistently fatal when the dosage was greater than 30 ml. per kilogram.

REFERENCES

1. Yamakawa, S.: Parenteral Nutrition, Part 1, Sec. B. Intravenous Fat Emulsions, Nippon Naika Gakkwai Zasshi **17**: 1, 1920.
2. Waddell, W. R., Geyer, R. P., Olsen, R. E., Andrus, S. B., and Stare, F. J.: The Clinical Experience With Intravenous Infusion of Emulsified Fat, J. Lab. & Clin. Med. **45**: 697, 1955.
3. Jordan, P. H., Jr.: Intravenous Administration of an Improved Fat Emulsion, Metabolism **6**: 656, 1957.
4. Artz, C. P.: Newer Concepts of Nutrition by the Intravenous Route, Ann. Surg. **149**: 841, 1959.
5. Watkin, D. M.: Clinical, Chemical, Hematologic and Anatomic Changes Accompanying Repeated Intravenous Administration of Fat Emulsion to Man, Metabolism **6**: 785, 1957.
6. Werr, J. A., and Preston, F. W.: Effect of an Intravenous Fat Emulsion on Blood Coagulation, A. M. A. Arch. Surg. **79**: 213, 1959.
7. Bozian, R. C., Davidson, N. W., Stutman, L. J., and Wilkinson, C. F.: Observations on the Use of Intravenous Fat Emulsions in Man, Metabolism **6**: 703, 1957.
8. Levenson, S. M., Upjohn, H. L., and Sheehy, T. W.: Two Severe Reactions Following the Long Term Infusion of Large Amounts of Intravenous Fat Emulsion, Metabolism **6**: 807, 1957.
9. Preston, F. W., Barnes, A. U., Mandel, E. E., Staley, C. J., and Trippel, O. H.: Effects of Repeated Infusions of a Fat Emulsion in Surgical Patients, Metabolism **6**: 758, 1957.
10. Singleton, W. S., White, J. L., Benerito, R. R., and Talluto, K. F.: Fat Emulsions: Effect of Polyoxyethylene and Alkyl Content of Emulsifiers on Stability to Sterilization, J. Am. Oil Chemists' Soc. **35**: 265, 1958.
11. Kern, F., Jr., Jackson, R. G., Martin, T. E., and Mueller, J. E.: Some Effects of Multiple Intravenous Infusions of a Cottonseed Oil Emulsion in Patients With Laennec's Cirrhosis of the Liver, Metabolism **6**: 743, 1957.
12. Herbst, F., Lever, W. F., and Waddell, W. R.: Effects of Intravenously Administered Fat on the Serum Lipoproteins, Science **123**: 843, 1956.
13. Shank, R. E., and Morrison, G.: A Review of Reactions After Long Term Intravenous Infusions of Fat (Lipomul I.V.), Proceedings of the Army Surgeon General's Task Force Group Conference on Intravenous Fat Emulsion, Vanderbilt University, School of Medicine, Nashville, Nov. 10-11, 1958, pp. 67-74.
14. Loewy, A., Freeman, L. W., Marchello, A., and Johnson, V.: Increased Erythrocyte Destruction on a High Fat Diet, Am. J. Physiol. **138**: 230, 1943.

15. Swank, R. L.: Changes in Blood of Dogs and Rabbits by High Fat Intake, Am. J. Physiol. **196**: 473, 1959.
16. Hartwig, Q. L., and Cohn, I., Jr.: Erythrocyte Sedimentation Rate and Blood Viscosity as Modified by Intravenous Fat Emulsions. In preparation.
17. Creditor, M. C.: Some Observations of Effects of Intravenous Fat Emulsion on Erythrocyte Fragility, Proc. Soc. Exper. Biol. & Med. **82**: 833, 1953.
18. Singleton, W. S., Benerito, R. R., Talluto, K. F., Brown, M. L., DiTrapani, L. L., and White, J. L.: Experiences With Prolonged Administration of Intravenous Fat Emulsions. Effects on Proteins of Dog Serum. In preparation.

STUDIES IN IRON ABSORPTION

I. Determination in Rats by Measurement of Total Body Radioactivity*

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NOT ONE of the methods currently in use for measuring iron absorption is entirely satisfactory. Determination of absorption by measuring fecal excretion of an oral dose of radioactive iron is cumbersome and subject to error in both the collecting and aliquoting of stools. Determining absorption from the fraction of an oral dose of radioactive iron incorporated into the red blood cells is unreliable because it depends upon a correct estimation of total blood volume and because an unknown fraction of the absorbed dose is deposited in the tissues.

Since Fe^{59} is a strong gamma ray emitter, the absorption of orally administered Fe^{59} can be conveniently measured by counting total body radioactivity. The application of this principle in rats is described in this article.

METHOD

A. *Description.*—The total body radioactivity in the rat is measured within a few hours after administration of an oral dose of radioiron, before any radioactivity has been excreted. Four to 7 days later, after sufficient time has elapsed for the unabsorbed part of the dose to be fully excreted, the total body radioactivity is measured again. After correcting for the physical decay of the isotope, the per cent absorbed is calculated from the ratio of the final to the initial count.

One milliliter of a solution which contains 10 to 20 μc of Fe^{59} as ferrous citrate and 0.1 to 0.3 mg. per kilogram† carrier iron in the ferrous form (adjusted to pH 4) is administered through a gastric tube; care is taken not to inject the solution into a bronchus. Within a few hours the rat is immobilized in a tight-fitting container to avoid counting rate variability caused by changes in body position. Cylindric specimen containers of various sizes have been used for part of this study. For rats of a uniform size, a special Lucite holder has been designed (Fig. 1). Total body radioactivity is then measured with the long axis of the rat aligned before a directional scintillation probe collimated with a 10-inch-long barrel which is insulated by a ⅞-inch-thick lead wall. The barrel gradually tapers from a 3½ inch diameter at its open end down to a 1 by 1 inch NaI thallium-activated crystal.

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**Dr. Field was a fourth-year medical student at Boston University when this work was carried out.

†Subsequent studies have revealed that greater reproducibility of results can be attained if a carrier dose of 2.5 mg per kilogram is employed, and there is apparently no resultant loss of sensitivity.

The number of counts per minute is recorded first with the tail and then with the head toward the probe, after which the counts are averaged. For the initial count, 2 minutes in each direction will usually suffice; the final count may require more time if the counting rate is low. A rat containing 1 μc of Fe^{59} and placed in the Lucite holder will record approximately 340 net counts per minute above a background of 100 c.p.m.

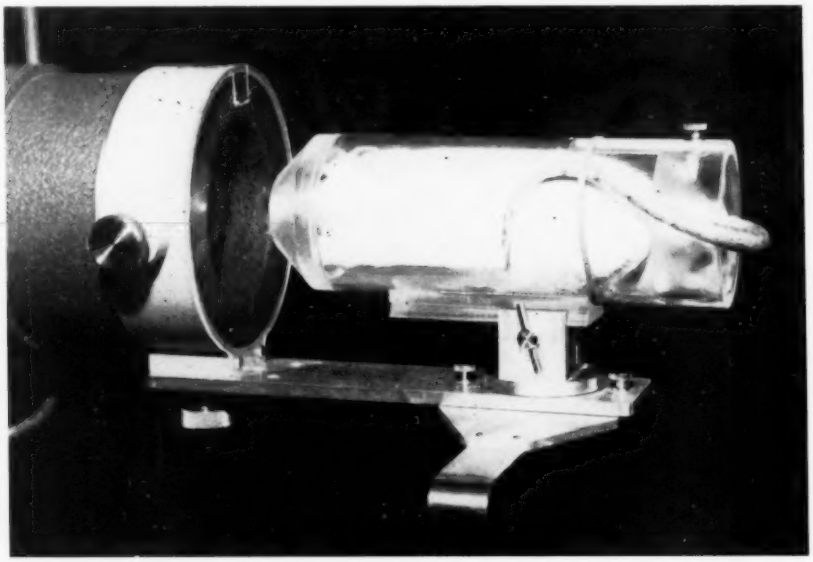

Fig. 1.—Rat holder in counting position. The rat is placed head first in the Lucite holder after the rear piece has been removed. When the rear piece is replaced, the rat's tail extends through the slit in the rear piece and is secured to the outside of the holder with a rubber band. There is a knob on top of the rear piece for adjusting it to fit a rat of any size. The inside of the holder is cone-shaped to hold the rat firmly in place. Under the rat is a pivot which allows the holder to be rotated, and on this is a wing bolt and a sliding track for adjusting the position of the holder so that the head and tail of either a large or a small rat will be approximately the same distance from the end of the barrel of the scintillation probe. The wing bolt also allows removal of the holder and will adjust to holders of different sizes. The arm extending off the bottom of the figure holds a plastic dish for collection of any urine that may be spilled. (The holder was manufactured by Laboratory Associates, Inc., Belmont, Mass.)

B. *Reproducibility and Reliability.*—The influence of the counting error on the calculated per cent of absorption was determined in 16 rats. Within 2 hours after an oral dose of Fe^{59} was given, each rat was counted once, removed from the holder for 30 minutes to 1 hour, and counted again. Duplicate counts were repeated 1 week later. Two separate per cents of absorption were calculated for each rat, one figure derived from the ratio of the first counts on each occasion and the other figure from the ratio of the second counts. Absorptions varying from 3.7 to 71.3 per cent had a mean deviation of 4.4 ± 1.0 per cent (± 1 C.V.; expressed as a per cent of the per cent absorbed).

Reproducibility will depend upon having somewhat similar counts when the head and tail of the rat are toward the counter. That adjustment of the position of the holder can minimize these differences is indicated by the following data. When 24 rats weighing approximately 400 grams were each given 10 μc of Fe^{59} by stomach tube and the 2 minute fore and aft counts were averaged, the mean of the mean counts was 2,881 ± 96 (± 1 S.D.). When the differences were averaged without regard to direction, the mean difference between fore and aft counts was 460 ± 289 (± 1 S.D.), or 16 ± 10 per cent. With regard to sign, the mean difference between counts was −24 ± 112 (± 1 S.E.), indicating no significant difference in direction between the fore and aft counts.

After 1 week the same rats averaged 359 c.p.m. above background and the mean difference between fore and aft counts was 18 ± 13 (± 1 S.D.), or 5 ± 3.6 per cent (± 1 C.V.). This time the head counts were significantly higher than the tail counts, the mean difference being + 11 ± 4 (± 1 S.E.). Thus, although the head and tail initial counts may vary by as much as 30 per cent, there is no directional trend, indicating that the differences probably caused by position of the stomach are cancelled out by counting from both the front and the rear. After 7 days the maximum variation is 12 per cent, but the head counts are significantly higher, probably a reflection of the distribution of blood within the body. Again, averaging the two minimizes the error.

The initial counts in this series also indicate that with the same dose administered there is a variation from rat to rat of 3 per cent (1 C.V.), a combination of the error of aliquoting and variations in the size of the rats, in addition to counting error. If the rats are of uniform size the initial count may be omitted and the final count related to a standard, but then the aliquoting error will increase in importance.

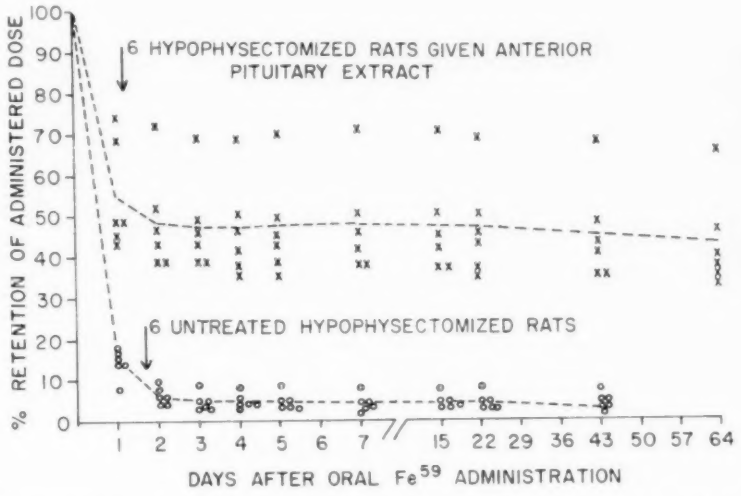

Fig. 2.—Changes in total body radioactivity (expressed as per cent of initial count) after a single oral dose of Fe^{59}.

Reliability of the method depends upon the rate of excretion of both nonabsorbed and absorbed radioiron. To determine this rate, twelve 200 gram hypophysectomized male Sprague-Dawley rats, 6 of which had been injected with anterior pituitary extract daily for the previous 10 days, were given Fe^{59} with a 50 mcg. carrier dose of ferrous ion, and total body counts were recorded daily for 1 week and at periodic intervals thereafter.*

The biologic decay curve for total body radioactivity shows an initial period of rapid decline which represents the excretion of nonabsorbed radioiron. A subsequent period of slow decline (1.10 ± 0.32 per cent [± 1 S.D.] of the amount present in hypophysectomized rats) probably represents the excretion of absorbed radioiron.† Therefore, the exact time chosen for the final count is not critical, so long as sufficient time has been allowed for excretion of nonabsorbed radioiron. Although a plateau was reached by the end of 4 days in all of the rats, fecal excretion may have been accelerated by the stress of daily body counting, and it is probably safer to employ a 6 or 7 day interval. The fact that a plateau is reached in 2

*Hypophysectomized rats were used in this experiment in order to eliminate changes in total body count resulting from increasing body size.

†The long-term excretion of absorbed radioiron in normal rats was not measured in this study. Stevens and co-workers[1] and Chapelle and associates[2] found an average daily iron excretion in normal mice of approximately 0.5 or 3.5 per cent per week.

to 4 days and that absorption at 7 days may be under 5 per cent (Fig. 2) indicates that coprophagy is an unimportant factor in the measurement of iron absorption when rats are kept in cages with wire net floors.

The existence of an almost negligible weekly excretion of radioiron after the first week (Fig. 2) makes it possible to perform repeated absorption studies with the same animals. A baseline body count is taken prior to administration of a new dose of radioiron. This value can then be subtracted from both the initial and final counts, because the level of residual radioactivity does not change appreciably in the time interval required for the new determination.

C. *Comparison of Three Methods for Measurement of Iron Absorption.*—After a single oral dose of Fe^{59}, absorption was measured in each of 18 rats by determining the radioactivity of the whole body, stools, and red blood cells.

One milliliter solutions which contained 10 to 15 μc of Fe^{59} as ferrous citrate and a carrier dose of 0.1 mg. of ferrous ion (as $FeSO_4 \cdot 7H_2O$) were administered through gastric tubes to eighteen 350 to 450 gram male Sprague-Dawley rats. Absorption was determined from initial and 7 day body counts.

TABLE I. COMPARISON OF THREE METHODS FOR MEASUREMENT OF IRON ABSORPTION (RATS ARRANGED IN ORDER OF DECREASING ABSORPTION AS DETERMINED BY TOTAL BODY COUNTING)

RAT NO.	CONDITION	I % ABSORBED AS DETERMINED BY TOTAL BODY COUNTING	II % ABSORBED AS DETERMINED BY STOOL COUNTING	III % ADMINISTERED DOSE INCORPORATED INTO CIRCULATING R.B.C.*	IV R.B.C. UTILIZATION ÷ % ABSORBED AS DETERMINED BY TOTAL BODY COUNTING (COLUMN III ÷ COLUMN I)
1	Blood loss, anemia	69.3	58.6	73.5	1.06
2	Blood loss, anemia	51.1	51.4	51.8	1.01
3	Blood loss, anemia	31.9	24.8	33.1	1.04
4	Blood loss, anemia	16.8	10.7	21.4	1.27
5	Normal	7.6	10.7	6.4	0.84
6	Normal	5.7	14.5	5.5	0.96
7	Normal	4.4	1.8	2.8	0.64
8	Iron loaded	4.0	0.6	1.8	0.45
9	Iron loaded	3.9	6.9	3.0	0.77
10	Normal	3.6	4.3	2.8	0.78
11	Normal	3.1	−0.2	1.4	0.45
12	Normal	3.0	0.6	2.3	0.77
13	Normal	2.9	3.8	2.5	0.86
14	Iron loaded	2.3	3.5	1.9	0.83
15	Iron loaded	1.3	9.9	1.2	0.92
16	Iron loaded	1.2	2.0	0.8	0.67
17	Iron loaded	0.5	−2.8	0.4	0.80
18	Iron loaded	0.5	3.2	0.4	0.80

*Calculated on the basis of a total blood volume of 5.1 per cent of body weight (in grams).[3]

One week stool collections from each rat were individually digested in concentrated sulphuric acid for 2 to 3 weeks. When indicated, stool digestion was accelerated by gentle heating and addition of 30 per cent hydrogen peroxide. In several instances the stool digestion mixtures were filtered over glass wool to attain a more suitable degree of homogeneity. The residue on the glass wool was washed with concentrated sulphuric acid and then with water. The washings were added to the original filtrates. Radioactivity of the residue on the glass wool was measured, and in no instance was it greater than 0.5 per cent of the administered dose. The radioactivity of weighed aliquots from the stool homogenates was measured in a conventional well-type scintillation detector. Two aliquots were taken from each homogenate, with an aliquote variation of 3.3 ± 2.9 per cent (± 1 C.V.).

After the final body count, each rat was anesthetized with ether, an abdominal incision was made, and blood was drawn into a heparinized syringe from the inferior vena cava. The radioactivity in 2 ml. blood samples was determined with the well-type scintillation detector. Total blood radioactivity was calculated on the basis of an assumed blood volume of 5.1 per cent of body weight (after Plzak and co-workers[3]).

In Table I the rats are listed in order from highest to lowest per cent of iron absorbed, as determined by total body counting. Three groups of rats are included: 4 rats made anemic by repeated bleedings, 7 normal rats, and 7 rats which had been fed diets supplemented with 6 per cent ferric citrate for 5 to 10 weeks.

All 3 methods of measurement bring out the difference in absorption between the anemic rats and the other 2 groups. A significant difference between the normal and the iron loaded rats, however, is brought out by the measurements of total body and red cell radioactivity, but not by measurements of stool radioactivity (Table II).

Figures for the per cent of the administered dose incorporated into circulating red cells correlate fairly closely with the figures for absorption determined by total body counting except in two instances (Rats 8 and 11). The per cent of the administered dose incorporated into red cells may be divided by the per cent absorbed, as measured by total body counting, to determine the fraction of the absorbed dose which enters into red cell formation. This fraction is significantly higher ($p < 0.001$) in the iron-deficient group (1.10 ± 0.10, $\pm$ 1 S.D.) than it is in either the normal (0.76 ± 0.16) or iron loaded group (0.75 ± 0.15).

TABLE II. DIFFERENCE IN IRON ABSORPTION BETWEEN IRON LOADED AND NORMAL RATS AS DETERMINED BY 3 METHODS

	% ABSORBED AS DETERMINED BY TOTAL BODY COUNTING		% ABSORBED AS DETERMINED BY STOOL COUNTING		R.B.C. UTILIZATION OF ADMINISTERED DOSE	
	NORMAL	IRON LOADED	NORMAL	IRON LOADED	NORMAL	IRON LOADED
Number of rats	7	7	7	7	7	7
Mean % absorbed	4.34	1.96	5.07	3.33	3.40	1.36
S. E. of mean	0.66	0.57	2.07	1.56	0.69	0.36
T (difference ÷ S.E. of difference)	2.7		0.67		2.6	
p	< 0.02		> 0.05		< 0.05	

DISCUSSION

Several methods of measurement have been employed in iron absorption studies. Prior to the introduction of radioisotope tracers into biologic research, absorption was measured by long-term iron balance studies. Although time consuming and difficult to perform accurately, these long-term studies had the advantage of measuring absorption of repeated doses of iron given over an extended period of time. Methods now in use are less representative because they measure absorption of only one dose of iron given at one particular moment.

Postabsorption serum iron curves are not satisfactory reflections of iron absorption. Changes in the serum iron level are partly determined by the rate at which iron is released from intestinal mucosal cells into the blood stream and partly by the rate at which iron passes from the serum to other tissues. This process may vary independently of the rate at which iron enters mucosal cells from the intestinal lumen.

Two methods which are commonly used for the measurement of iron absorption have been compared to the technique of total body counting with regard to accuracy and reliability. Measurement of fecal excretion of radioiron

was found to be less accurate than total body counting at low, but physiologic, levels of absorption (Table II). When iron absorption is low, a small error in the measurement of the excretion results in a large error in the calculated absorption. For example, if the true excretion of an oral dose is assumed to be 95 per cent with a 5 per cent error in the determination, then the measured per cent absorbed will vary from 0 to 10 per cent, which obviates any comparisons at such low levels of absorption. When absorption is measured directly by total body counting, a 5 per cent error in the measurement of a true absorption of 5 per cent would result in a value ranging between 4.5 and 5.5 per cent. Body counting is also likely to be more accurate than stool counting, even at higher levels of absorption, because it eliminates the error in administering the dose and in collecting and aliquoting stools.

Red cell utilization of an oral dose of radioiron depends upon both the per cent of the administered dose absorbed and the per cent of the absorbed dose incorporated into circulating red cells. Therefore, measurements of red cell radioactivity cannot be accepted as reliable estimates of iron absorption under varying physiologic conditions unless reasonable assurance is offered that the fraction of absorbed iron incorporated into red cells is constant for the different conditions under study. Lack of such assurance constitutes the principal objection to red cell utilization as a measure of absorption.

The fraction of absorbed iron incorporated into red cells is significantly higher in rats with anemia caused by iron deficiency than it is in normal rats (Table I). Although no difference in this fraction between normal and iron loaded rats was demonstrated in this study, depressed utilization of absorbed iron in the presence of excess iron stores has been noted by others.[4] Dubach and associates[5] found the red cell utilization of intravenously injected radioiron to vary considerably under different hematologic conditions. Variations in the red cell utilization of absorbed radioiron have not been adequately studied, probably because there has been no sufficiently accurate method for measuring absorption.

Saylor and Finch[6] have used a double isotope technique to control this variable by determining the red cell utilization of an intravenous dose of radioiron with the assumption that utilization of a simultaneously absorbed oral dose will be the same. However, the assumption that there is equivalent red cell utilization of radioiron, whether it is given by the oral or intravenous route, needs to be substantiated.

Total body counting appears to be the best available method for the measurement of the absorption of single doses of radioiron, but the technique has been applied only to rats. Preliminary studies are underway to determine the feasibility of measuring iron absorption in humans by a previously described technique of total body counting.[7]

SUMMARY

1. Iron absorption can be measured in the rat by counting total body radioactivity immediately after administration of radioiron and 4 to 7 days later.

The second figure divided by the first figure, multiplied by 100, with correction for the physical decay of the isotope, represents the per cent absorbed.

2. When absorption is measured by utilizing a tight-fitting rat holder and a directional scintillation probe, the mean error in duplicate determinations is 4.4 per cent at absorptions between 3.7 and 71 per cent. The importance as a source of error of differences in the distribution of Fe^{59} within the rat is minimized by counting each rat from the front and rear, and averaging the results.

3. In a series of 16 rats, full excretion of the nonabsorbed radioiron had occurred by the end of 6 days in every instance. Continued excretion of the absorbed radioiron proved to be gradual, occurring at the rate of 1.1 per cent per week in hypophysectomized rats.

4. Iron absorption was measured in iron-deficient, normal, and iron loaded rats by total body counting, stool counting, and red cell counting. Greater than normal efficiency of absorption was demonstrated in anemic rats by all 3 methods, whereas less than normal efficiency of absorption was demonstrated in rats with increased iron stores by total body and red cell counts, but not by stool counting.

5. The fraction of absorbed iron incorporated into circulating red cells by the end of a week was significantly greater in iron-deficient than in normal or iron loaded rats. No difference in this fraction between the latter 2 groups was found.

REFERENCES

1. Stevens, A. R., Jr., White, P., Hegsted, D. M., and Finch, C. A.: Iron Excretion in the Mouse, J. Biol. Chem. **203**: 161, 1953.
2. Chapelle, E., Gabrio, B. W., Stevens, A. R., and Finch, C. A.: Regulation of Body Iron Content Through Excretion in the Mouse, Am. J. Physiol. **182**: 390, 1955.
3. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F.: Demonstration of Stimulation of Erythropoiesis by Plasma From Anemic Rats Using Fe^{59}, J. Lab. & Clin. Med. **46**: 671, 1955.
4. Josephs, H. W.: Absorption of Iron as a Problem in Human Physiology. A Critical Review, Blood **13**: 1, 1958.
5. Dubach, R., Moore, C. V., and Minnich, V.: Studies in Iron Transportation and Metabolism. V. Utilization of Intravenously Injected Radioactive Iron for Hemoglobin Synthesis, and Evaluation of Radioactive Iron Method for Studying Iron Absorption, J. Lab. & Clin. Med. **31**: 1201, 1946.
6. Saylor, L., and Finch, C. A.: Determination of Iron Absorption Using Two Isotopes of Iron, Am. J. Physiol. **172**: 372, 1953.
7. Hine, G. J., and Burrows, B. A.: Total and Partial Body Radioactivity Measurements for Metabolic Tracer Studies, Proceedings of the Second International Conference on Peaceful Uses of Atomic Energy, Geneva, 1958.

A STUDY OF THE CARRIER STATE FOR PLASMA THROMBOPLASTIN COMPONENT (PTC, CHRISTMAS FACTOR) DEFICIENCY, UTILIZING A NEW ASSAY PROCEDURE

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THE diagnosis and treatment of plasma thromboplastin component (PTC) deficiency has been greatly facilitated by the use of such newer blood coagulation methods as the thromboplastin generation test[1] and the partial thromboplastin time test.[2] These tests are qualitative, however, and the accurate quantitative measurement of PTC has been a serious problem. Prior to the development in our laboratory of the PTC assay,[3] the method most widely employed had been the corrective effect of dilutions of serum in the thromboplastin generation test.[4] While it is true that serum deficient in PTC will not "generate thromboplastin" normally, this is also true of serum deficient in Stuart factor.[5] Therefore, direct inferences from the per cent thromboplastin generated to per cent PTC present must be viewed with skepticism.

We shall describe in detail an assay procedure in which all factors, with the exception of PTC, are present in adequate amounts. The method is, for this reason, specific and reasonably accurate. The systematic application of this procedure to a study of the carrier state for PTC deficiency constitutes the body of this article.

MATERIALS

Imidazole buffer was prepared by dissolving 1.72 Gm. of imidazole, C.P., in 90 ml. of 0.1N hydrochloric acid. The pH was adjusted to 7.2, and the volume was brought to 100 ml. with distilled water.

Buffered sodium citrate was prepared by dissolving 3.2 Gm. of trisodium citrate in imidazole buffer; the volume was brought to 100 ml. with the buffer. Unbuffered *sodium citrate solution* was prepared by dissolving 3.2 Gm. of trisodium citrate in distilled water; the volume was brought to 100 ml.

Buffered citrated saline was prepared by mixing 100 ml. of 0.9 per cent sodium chloride, 100 ml. 1.72 per cent imidazole buffer, pH 7.2, and 40 ml. 3.2 per cent trisodium citrate solution.

Cephalin was prepared as described by Rodman and co-workers.[2] The 0.3 per cent saline suspension was used for the partial thromboplastin time tests and the 0.015 per cent suspension was used for the PTC assay.

Calcium chloride, 0.02M, was prepared for use in the partial thromboplastin time test.

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Calcium solution for use in the PTC assay was prepared by mixing 70 ml. of 1.2 per cent calcium chloride, 60 ml. of imidazole buffer, pH 7.2, and 50 ml. of 0.9 per cent sodium chloride.[6]

Control Plasmas.—Blood was drawn by the 2 syringe technique from 89 female students of nursing ranging in age from 17 to 24 years. Eight volumes of blood were mixed with 1 volume of buffered sodium citrate. After centrifugation at 3,200 g for 15 minutes, plasma was withdrawn and divided into 2 portions. A 2 ml. aliquot was lyophilized immediately. The remainder was frozen and stored for several days at -20° C. After all 89 plasmas had been obtained, the individually frozen samples were thawed, pooled, and lyophilized. This pooled, freeze-dried plasma was used as the control for all PTC assays. The individually lyophilized plasmas of the normal girls formed the normal control population, and each was assayed separately. Sixty-one of the samples were reassayed 2 months later, the other 28 samples having been exhausted. The normal population used for statistical purposes in defining the normal range for PTC consisted of the average of the 2 values on each of the 61 girls on whom 2 PTC assays had been done.

Test Plasmas.—One of us (W. R. B.) visited the homes of the men with PTC deficiency to collect blood for testing. Blood was drawn from affected males and their mothers by the 2 syringe technique. Eight volumes of blood were placed in a conical centrifuge tube which contained 1 volume of buffered citrate solution. Plasma was prepared by centrifugation at 3,200 g, was withdrawn, quickly frozen at -70° C., and brought to the laboratory. A partial thromboplastin time was performed on a sample of thawed plasma from each of the sons. The remainder of the plasma from each male and all of the plasma from his mother was shell-frozen and lyophilized. PTC assays were performed at a later time on reconstituted lyophilized plasma from each mother and each son. All blood samples for the study were obtained during a period of 2 months.

PTC Deficient Substrate.—Lyophilized plasma was prepared from a patient (J. J. T.) with severe PTC deficiency who had been previously studied in our laboratory.[2] His dried plasma was reconstituted as needed and was diluted immediately before use with an equal quantity of fresh barium sulfate adsorbed canine plasma; thus, adequate amounts of antihemophilic factor (AHF), factor V, and Hageman factor were supplied.

Reconstituted Plasma.—All lyophilized plasmas (test, normal, pooled control, and substrate) were reconstituted with imidazole buffer and brought to the original plasma volume.

$BaSO_4$ adsorbed canine plasma was prepared by mixing 100 mg. of $BaSO_4$ powder* with each milliliter of oxalated canine plasma. The plasmas were adsorbed for 30 minutes at 5° C. with frequent stirring. The $BaSO_4$ was removed by centrifugation and the supernatant plasma was used in the assay after it was ascertained that no clot formed in 10 minutes in the standard prothrombin time test.

METHODS

The *partial thromboplastin time* test of Langdell and associates[6] as described by Rodman and co-workers[2] was performed on plasmas from the 17 sons. Testing was done either on plasmas freshly prepared in the laboratory, or on plasmas which had been brought to the laboratory in the frozen state, and had then been thawed.

The *PTC assay* is an adaptation of the AHF assay described by Langdell and associates.[6] The PTC activity is expressed as per cent of control by comparing the effectiveness of control and unknown plasmas in correcting a plasma known to be deficient only in PTC. The assay is performed as follows:

All reagents are kept in a water bath at 28° C. during testing. (The PTC-deficient substrate may be kept at 0° C. as in the AHF assay, but this does not appear to be as crucial.) One per cent, 2.5, 5, 10, and 20 per cent concentrations of control plasma are prepared with buffered citrated saline. Only 3 concentrations of test plasma are made. (The

*Merck & Co., Inc., Rahway, N. J.

choice of dilutions of test plasma depends upon the value expected from a preliminary partial thromboplastin time test on the unknown plasmas.) Each diluted plasma sample is tested by mixing the reagents in the following order: 0.1 ml. PTC substrate, 0.1 ml. diluted control or test plasma, 0.1 ml. 0.015 per cent cephalin suspension, and 0.1 ml. calcium-imidazole-saline solution.

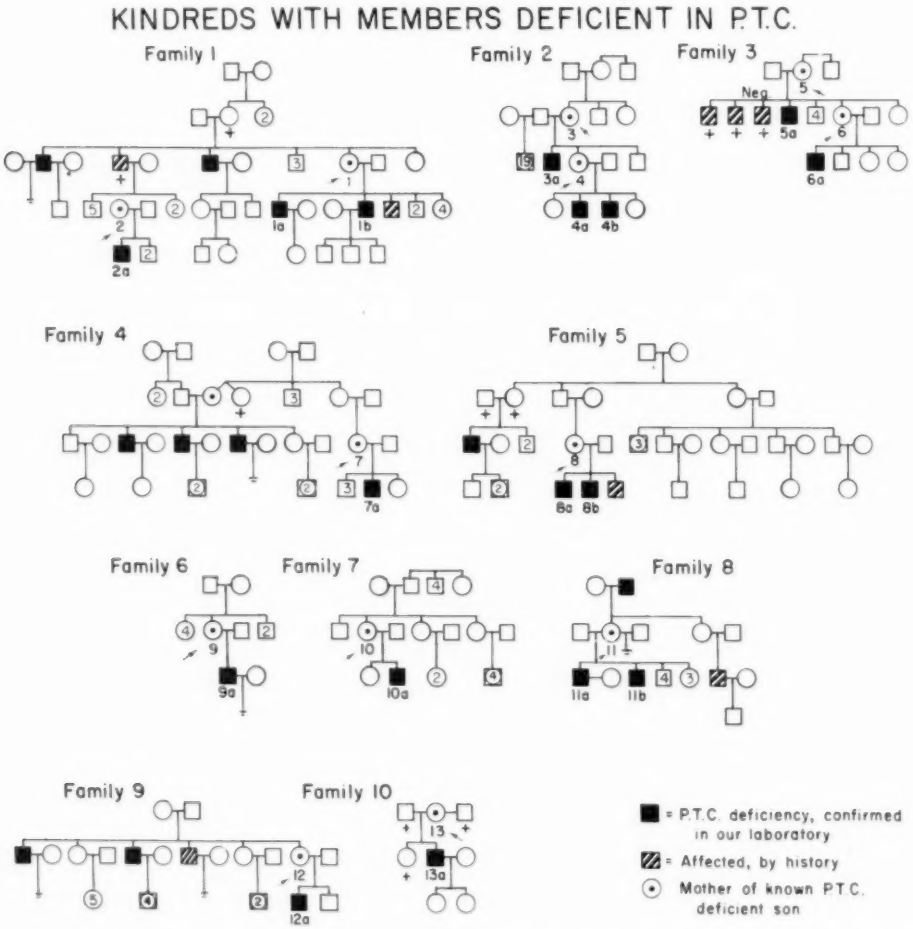

Fig. 1.—Partial pedigree charts of the 10 families selected for study. Some families contain only 1 affected male while others have 2 or more. Families *1*, *3*, *4*, *5*, *8*, and *9* contain additional affected males not included in our investigation. Son number *6a* died from hemorrhage after the completion of the study.

A stop watch is started with the addition of the calcium solution, and the clotting time for each dilution is recorded. As many as 6 assays may be run simultaneously, 5 unknowns and 1 control. The dilutions are tested in order (i.e., all of the 20 per cent plasmas in the test block, then the 10 per cent plasmas, etc.). The procedure is repeated in the same order after about 45 minutes so that duplicate determinations are available on each dilution of each plasma. The duplicate clotting times at each dilution are averaged and plotted against the logarithm of plasma concentration, using 2 cycle, semilogarithmic paper. The best fitting parallel straight lines are fitted to the values for the unknown and the control, and the concentration of test plasma with respect to control plasma is determined by interpolation as in

the AHF assay. Over the concentration range used, there is usually less variation about the lines plotted than in the AHF assay.

RESULTS

The PTC assay has been applied systematically in a study of the carrier state in PTC deficiency. The family information on which this project depended (Fig. 1) was obtained by clinical and laboratory investigation of 13 mothers and 17 of their affected sons in 10 families with PTC deficiency. More extensive investigation of these families than is shown in Fig. 1 did not uncover any relationship between the families.

Variation in PTC Levels in a Normal Population.—The PTC assay procedure was used to define the range of PTC levels in normal women, a necessary step prior to any decision about the effect of the abnormal PTC gene in carriers.

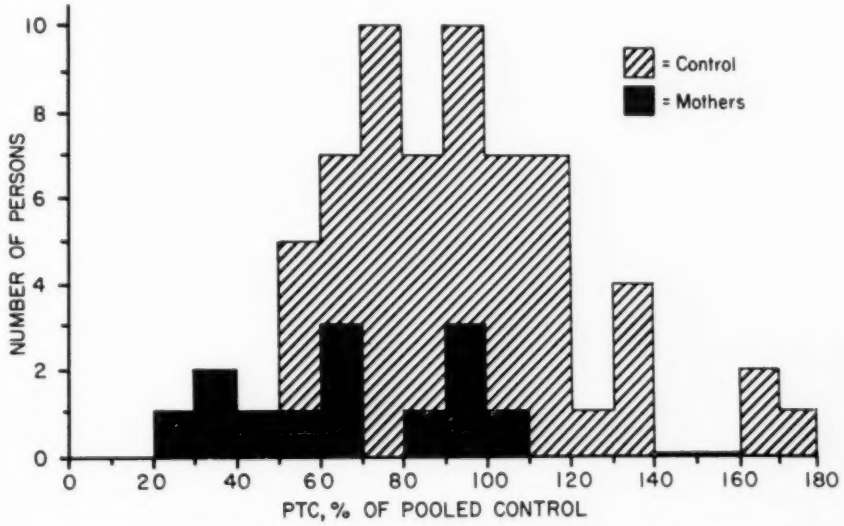

Fig. 2.—A frequency distribution of the control population of 61 student nurses and the experimental population of 13 mothers whose sons have PTC deficiency. The number of persons with each increment of 10 per cent in PTC is charted on the ordinate. The PTC levels in increments of 10 per cent of the pooled control are plotted on the abscissa.

Table I contains the actual PTC values of the normal women and the statistics which describe the normal population. The frequency distribution of the population shown in Fig. 2 appears to be roughly gaussian. The mean PTC level expected in the normal population was 100 per cent because of the nature of the control. The mean PTC level observed was 93 per cent of this pooled control.

Variation in Severity of PTC Deficiency in Affected Sons.—The partial thromboplastin times, the levels of PTC, and the clinical severity of each affected son are recorded in Table II. A wide range of coagulation defects and symptoms is apparent. It should be noted that there is a fairly good correlation between prolongation of the partial thromboplastin time, reduction in PTC, and clinical severity.

Comparison of PTC Levels in Carriers and Normal Controls.—The PTC levels of the carriers which are recorded in the third column of Table II are shown also as a frequency distribution in Fig. 2. The group mean for the carriers was 67 per cent, and they appear to form a bimodal distribution. The mean PTC level of the carrier population was found to be significantly lower than the mean PTC level of the control population by a "t" test. The value for "t," assuming the null hypothesis, was 3.19. With 72 degrees of freedom, a value this large would be expected less than 1 per cent of the time by chance alone if the 2 populations were actually subsamples of the same larger population.

TABLE I

PTC LEVELS OF 61 NORMAL YOUNG WOMEN				
171	110	95	84	71
166	110	94	81	69
165	110	93	80	68
138	109	93	79	65
135	107	91	78	64
132	107	91	78	61
131	107	90	78	61
126	106	90	77	60
116	105	89	77	58
115	100	88	75	57
114	97	86	73	56
112	97	84	71	56
				55

These values were obtained by averaging duplicate determinations of PTC obtained 2 months apart on the same samples of lyophilized plasma. A pooled plasma from the group was the 100 per cent standard. The expected mean for this group was, therefore, 100 per cent. The observed (calculated) mean was 93 per cent with 95 per cent fiducial limits of 86 to 100 per cent. Standard deviation was 27 per cent and standard error was 3.5 per cent.

Laboratory Diagnosis of the Carrier State.—Using the statistics derived from the normal population (Table I) a discriminant can be devised for detection of an abnormally low PTC value in a woman suspected of being a carrier for PTC deficiency. The discriminant can be set at the 95 per cent confidence point by subtracting 2 standard deviations from the mean of the control population, i.e., 93 – 54 = 39 per cent. Thus any woman with a PTC level lower than 39 per cent is highly suspect. The utility of the discriminant can be observed by noting that 3 of the known carriers in Table II were exactly 39 per cent or lower; it should also be observed that the other 10 mothers had values higher than 39 per cent.

Clinical Symptoms in Carriers.—The mothers were queried closely to determine the extent of a bleeding tendency, because it has been generally believed that carriers for PTC deficiency bleed excessively. We found that not one had sought medical help for treatment of hemorrhage or for diagnosis of a coagulation defect. They were known to be carriers only because they had had affected sons. The ordinary occasions when these women might have been expected to hemorrhage were collated and are shown in Table III. With the one exception noted, no mother had required transfusions to stanch excessive bleeding in spite of PTC levels as low as 29 per cent.

Inheritance of Severity of PTC Deficiency.—The relationship between the PTC level of a mother and the PTC level of her affected son (or sons) is plotted

in Fig. 3. The chart is divided into 4 cells (*A*, *B*, *C*, and *D*) by the intersection of the vertical and horizontal broken lines. The vertical line at 67 per cent represents the mean PTC for the group of women, a value which divides them equally into high and low groups. The horizontal line at 5 per cent represents the lower limit of accurate measurement for the assay procedure. The more seriously affected sons have levels of PTC which are less than 5 per cent but are not measurable with any accuracy. (It can be seen very clearly in Table II that the partial thromboplastin time is a better index of clinical severity than the PTC assay when the PTC level is less than 5 per cent.)

If the PTC level of a mother were unrelated to that of her son, an equal number of sons with PTC levels greater than 5 per cent would be expected to fall in cells *A* and *B*, and an equal number of sons with PTC levels less than 5

TABLE II

FAMILY NO.	MOTHERS		SONS				
	MOTHER NO.	PTC* (%)	SON NO.	SON INITIALS	PARTIAL THROMBOPLASTIN TIME (SECONDS) PATIENT/CONTROL	PTC, % POOLED CONTROL	CLINICAL SEVERITY†
1	1	87	1a	H. W.	238/78	< 5	Moderate
1	1	87	1b	R. W.	189/81	10	Mild
1	2	92	2a	M. F.	152/73	8	Mild
2	3	93	3a	W. S.	284/90	< 5	Severe
2	4	60	4a‡	W. B.	638/82	< 5	Severe
2	4	60	4b	D. B.	411/89	< 5	Severe
3	5	49	5a	J. T.	218/82	< 5	Severe
3	6	54	6a§	C. M.	330/90	< 5	Severe
4	7	60	7a	D. F.	126/75	8	Moderate
5	8	109	8a	G. P.	84/70	59	Mild
5	8	109	8b	W. P.	118/70	33	Mild
6	9	39	9a	H. A.	256/105	< 5	Moderate
7	10	29	10a	E. Y.	450/83	< 5	Severe
8	11	35	11a	C. H.	167/77	< 5	Moderate
8	11	35	11b	J. H.	155/77	< 5	Moderate
9	12	68	12a	E. L.	123/70	5	Mild
10	13	96	13a	A. C.	170/81	9	Moderate

The laboratory results on the 13 carrier mothers and 17 affected sons from 10 families segregating for PTC deficiency. The numbers refer to families and individuals shown in the pedigree charts of Fig. 1. The mothers' PTC levels ranged from 29 to 109 per cent with a mean of 67 per cent, 95 per cent fiducial limits of 83 and 51 per cent, standard deviation of 26 per cent, and standard error of 7.2 per cent.

*Average of 2 determinations on lyophilized sample. Lyophilized pooled control = 100 per cent.

†A male who reported apparently spontaneous hemarthroses was scored clinically as *severe;* one who reported hemarthroses only after obvious and severe trauma was scored *moderate;* one who reported never having hemarthroses despite athletic activity was scored *mild.*

‡Developed circulating anticoagulant after transfusion on 3 separate occasions.[2] This study performed during remission.

§Died after study was completed.

TABLE III. AMOUNT OF PHYSICAL TRAUMA OF SIMILAR TYPE EXPERIENCED BY 13 HETEROZYGOUS MOTHERS

CHILDREN (NO.)	MAJOR OPERATIONS (NO.)	TEETH EXTRACTED (NO.)	TRANSFUSIONS (NO.)
53	11	198	4

Only Mother 9 (Family 6) had transfusions. She had been given 4 units of whole blood on 1 occasion, during and after a radical mastectomy.

per cent would be found in cells *C* and *D*. This proved *not* to be the case as shown in Fig. 3. Rather, the points are concentrated in cells *B* and *C*. Only 1 mother of 7 with a PTC level less than 67 per cent had a son with more than 5 per cent PTC, while the 6 other mothers had 8 sons with PTC levels less than 5 per cent. From the group of 6 carriers with PTC levels greater than 67 per cent, 6 of 8 sons had levels of PTC greater than 5 per cent whereas 2 had levels of PTC less than 5 per cent. These findings were tested by a heterogeneity chi square to determine the significance of the distortion from a random distribution using the Yates correction for continuity. Chi square was 6.98 which, with 1 degree of freedom, means that distortion of this degree would be expected less than 1 per cent of the time by chance alone.

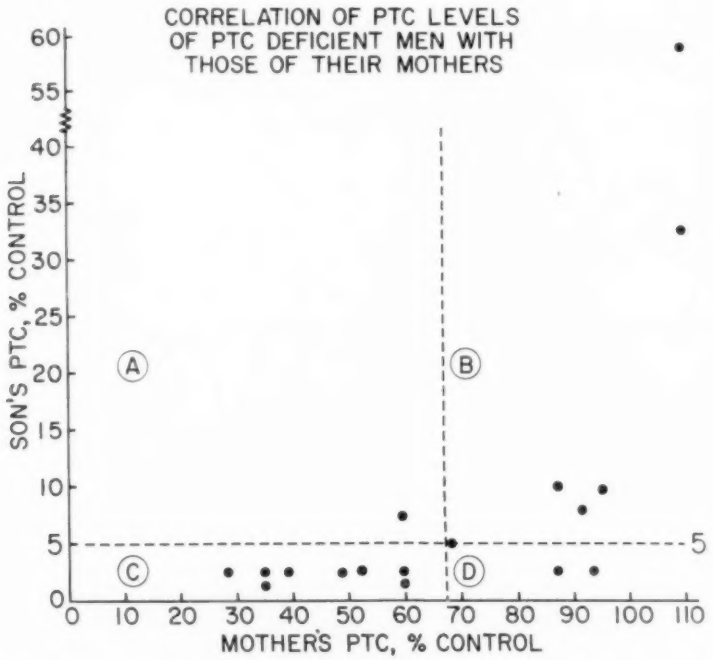

Fig. 3.—A plot of the level of PTC of each mother against that of her affected son (or sons). Each point represents a mother-son combination. The vertical broken line divides the carriers into those below and above the observed mean for mothers (67 per cent). The sons are divided by the horizontal broken line into those with PTC levels greater than, or less than, 5 per cent. The chart is divided by the intersection of the vertical and horizontal lines into 4 cells labeled *A*, *B*, *C*, and *D*. The significance of this distribution is discussed in the text.

DISCUSSION

The assay which we have described appears to be a reliable procedure for the determination of plasma PTC levels. It is essential that a suitable control be established before the assay is used as a regular laboratory procedure. Since we have shown that there is a wide variation in a normal population, it is recommended that a pooled control be used as the 100 per cent standard. Lyophilization will provide a control suitable for periods up to 1 year, particularly if the dried plasma is stored at –20° C. or lower.[7] We have observed a consistent loss of about 20 per cent PTC as a result of lyophilization.

Some of the fundamental genetic aspects of PTC deficiency have been illuminated by the application of this assay procedure.

1. The carrier state in PTC deficiency has been investigated several times[8-13] since the disease was first reported.[14-16] Previous studies of families with affected members have shown that the defect is transmitted as a sex-linked recessive.[8, 9, 17-19] It has been suggested that PTC deficiency is not as completely recessive as hemophilia because several women, who are transmitters, have been reported to have abnormal clotting tests.[8, 9, 13, 20-23] Our study clearly shows that the PTC level of heterozygotes *as a group is lower than that of normal women.* This means that heterozygosity *does* have an effect on PTC level, and that the abnormal gene must be considered not completely recessive. Furthermore, approximately 1 transmitter in 4 was found to have a PTC level low enough for her to be scored as abnormal with confidence. Nevertheless, the great majority do not clearly have abnormally low PTC levels.

2. Regarding the question of a significant hemorrhagic tendency in women who are carriers, our data show that they withstand considerable trauma without difficulty. This raises a question for which we have no facile answer. Why does a heterozygous woman with a PTC level of 29 per cent not bleed abnormally, whereas hemizygous boys with 33 and 59 per cent PTC levels bleed sufficiently to bring them to our laboratory? The absence of a hemorrhagic tendency in the carriers with low PTC levels suggests a protective influence on the vascular tree of women independent of PTC. This influence may be the female sex hormones, because it is known that estrogens are effective in curbing hemorrhagic episodes and in increasing plasma Ac-globulin.[24]

3. In examining the relationship between PTC levels of hemizygous sons and their heterozygous mothers, we find that not only is the *deficiency* of PTC inherited, but also the *severity.* There is a highly significant correlation between the PTC levels of affected sons and the reduced PTC levels of carrier mothers. From the genetic standpoint this suggests at least 2 hypotheses. First, there may be a number of alleles capable of residing at the PTC locus which have different efficiencies in controlling PTC levels. On the other hand the gene pool may contain only 1 normal and 1 abnormal allele for this locus, while modifier genes elsewhere in the genome determine the variability in a population of affected males. In either instance, a mother-son correlation would be expected. Our data are not definitive in making a choice between these alternatives, but tend to support the concept of multiple alleles.

The plot of PTC levels of the heterozygotes in Fig. 2 suggests a bimodal distribution. This may be considered evidence strengthening the hypothesis of 2 or more abnormal PTC alleles. (The observation, of course, may simply represent a sampling error, because 13 mothers do not constitute a very large sample for statistical purposes.) Another intrafamily correlation not apparent in Fig. 3 probably adds some slight additional weight to the multiple allele hypothesis. Among the affected boys, 4 sets of brothers were tested and plotted. Three of the 4 pairs lay in the same cells; only 1 pair was divided between 2 of the cells. If modified genes were very important, both mildly and severely affected hemizygous boys would be expected within a sibship.

A recent experience with a family, not a part of the present study, adds more weight to the importance of multiple alleles in PTC deficiency. The family consisted of a grandfather with moderately severe PTC deficiency (< 5 per cent PTC), 2 carrier daughters, and 3 sons of the 2 daughters. All 3 of the grandsons had PTC levels less than 5 per cent and clinical symptoms either equal to or more severe than those of their grandfather. One transmitter daughter had a PTC level of 100 per cent, while the other had a level of 29 per cent. Both daughters obviously received the same abnormal PTC allele from the father and transmitted it to their sons. Yet the PTC levels of these sisters were significantly different. The difference between the sisters may have resulted from the allele on the PTC locus of the other X-chromosome, because it must be remembered that each had a normal allele contributed by the mother. Their normal mother may have transmitted alleles of quite different efficiency to each daughter. While multiple allelism is a likely explanation of the foregoing, our data do not completely exclude the possibility that modifier gene(s) at some other locus on the X-chromosome or on an autosome were responsible for this interesting family. A somewhat analogous situation has been described by McGovern and Steinberg[25] in AHF deficiency.

The preceding statements should not be taken to mean that acceptance of either hypothesis excludes the possibility of the other. We have set up the mutually exclusive hypotheses for heuristic purposes. It is entirely possible that the PTC locus may both be occupied by one or another of a series of alleles and at the same time subject to modification by other genetic loci.

SUMMARY

1. An assay method for measuring PTC levels, based on the partial thromboplastin time technique of Langdell and co-workers,[6] is described in detail.

2. The PTC levels of a control female population, a group of 13 heterozygous carrier mothers, and 17 affected sons are described and compared. A good correlation was observed between clinical severity, PTC levels, and prolongation of clotting tests of the affected males.

3. This study has produced the following genetic information about the mutant gene(s) for PTC deficiency:

A. Heterozygosity, as measured by group means, causes a slight but significant reduction in PTC, which implies that the mutant gene should not be considered completely recessive.

B. Even with a significant *average* reduction in PTC level, heterozygous females withstand considerable trauma without dangerous loss of blood.

C. In 25 per cent of the heterozygotes studied, PTC was sufficiently reduced to allow a confident diagnosis of abnormality by our assay. However, the PTC levels of 75 per cent of the mothers fell within the normal range.

D. The severity of PTC deficiency is clearly inherited. However, it is not clear whether this is due to a multiple allelic series at this locus, a single abnormal allele greatly influenced by the remainder of the genome, or both. Our present data tend to indicate a multiple allelic series at the PTC locus. However, it would be a very unusual locus which was completely independent of the rest of the genome.

We would like to express our appreciation to the Faculty and students of the School of Nursing of the University of North Carolina for their participation in this study.

REFERENCES

1. Biggs, R., and Douglas, A. S.: The Thromboplastin Generation Test, J. Clin. Path. **6:** 23, 1953.
2. Rodman, N. F., Barrow, E. M., and Graham, J. B.: Diagnosis and Control of the Hemophilioid States With the Partial Thromboplastin Time (PTT) Test, Am. J. Clin. Path. **29:** 525, 1958.
3. Graham, J. B., Bullock, W. R., and Barrow, E. M.: The Characteristics of the Carrier State in PTC-Deficiency (Christmas Disease), Proceedings of the VII Congress of the International Society of Hematology, 1958. In press.
4. Barkhan, P.: Christmas Factor Activity of Cord Blood, Brit. J. Haemat. **3:** 215, 1957.
5. Hougie, C., Barrow, E. M., and Graham, J. B.: Stuart Clotting Defect. I. Segregation of an Hereditary Hemorrhagic State From the Heterogeneous Group Heretofore Called "Stable Factor" (SPCA, Proconvertin, Factor VII) Deficiency, J. Clin. Invest. **36:** 485, 1957.
6. Langdell, R. D., Wagner, R. H., and Brinkhous, K. M.: Effect of Antihemophilic Factor on the One-Stage Clotting Tests, J. Lab. & Clin. Med. **41:** 637, 1953.
7. Geratz, J. D., and Graham, J. B.: The Efficacy of Plasma Reclaimed From Outdated Blood in the Treatment of Plasma Thromboplastin Component (PTC) Deficiency, Proc. Internat. Soc. Blood Trans., 1958, Bibl. haemat., Basel: 993, 1959.
8. Ramot, B., and Angelopoulos, B.: Variable Manifestations of Plasma Thromboplastin Component Deficiency, J. Lab. & Clin. Med. **46:** 80, 1955.
9. Didisheim, P., Ferguson, J. H., and Lewis, J. H.: Hemostatic Data in Relatives of Hemophiliacs A and B, A. M. A. Arch. Int. Med. **101:** 347, 1958.
10. Rosenthal, R. L.: Hemophilia and Hemophilia-Like Diseases Caused by Deficiencies in Plasma Thromboplastin Factor, Am. J. Med. **17:** 57, 1954.
11. Bergsagel, D. E., Setna, S. S., Cartwright, G. E., and Wintrobe, M. M.: Mild PTC (Plasma Thromboplastin Component) Deficiency Occurring in Two Brothers, Blood **9:** 866, 1955.
12. Firkin, B. G.: The Demonstration of the Carrier State in Christmas Disease, M. J. Australia **1:** 557, 1958.
13. Moor-Jankowski, J. K., Truog, G., und Huser, H. J.: Der Bluterstamm von Tenna und seine Nachkommen, Acta genet., Basel **7:** 1, 1957.
14. Aggeler, P. M., White, S. G., Glendening, M. B., Page, E. W., Leake, T. B., and Bates, G.: Plasma Thromboplastin Component (PTC) Deficiency: A New Disease Resembling Hemophilia, Proc. Soc. Exper. Biol. & Med. **79:** 692, 1952.
15. Biggs, R., Douglas, A. S., Macfarlane, R. G., Dacie, J. V., Pitney, W. R., Merskey, C., and O'Brien, J. R.: Christmas Disease: A Condition Previously Mistaken for Hemophilia, Brit. M. J. **2:** 1378, 1952.
16. Schulman, T., and Smith, C. H.: Hemorrhagic Disease in an Infant Due to Deficiency of a Previously Undescribed Clotting Factor, Blood **7:** 794, 1952.
17. Cramer, W., Flückiger, P., Gasser, C., Koller, F., Loeliger, A., and Matter, M.: Two Cases of Hereditary Hemophilia Due to a Deficiency of a New Clotting Factor (Christmas factor), Acta Haemat. **10:** 65, 1955.
18. Lewis, J. H., and Ferguson, J. H.: Hemorrhagic Diathesis Due to PTC (Plasma Thromboplastin Component) Deficiency, Proc. Soc. Exper. Biol. & Med. **82:** 445, 1953.
19. Rosenthal, M. C., and Sanders, M.: Plasma Thromboplastin Component Deficiency, Am. J. Med. **16:** 153, 1954.
20. Brinkhous, K. M., Langdell, R. D., Penick, G. D., Graham, J. B., and Wagner, R. H.: Newer Approaches to the Study of Hemophilia and the Hemophilioid States, J. A. M. A. **154:** 481, 1954.
21. Hardisty, R. M.: Christmas Disease in a Woman, Brit. M. J. **1:** 1039-1040, 1957.
22. Hule, V., Nešpůrková, M.: Angeborener Faktor IX-Mangel (Christmas)—Hämophilie B, Ann. paediat. **186:** 175, 1956.
23. Bond, T., Celander, D. R., and Guest, M. M.: The Detection of Plasma Thromboplastin Component (PTC) Deficiency Carriers, Fed. Proc. **18:** 14, 1959.
24. Johnson, J. F.: Changes in Plasma Prothrombin, Ac-Globulin, and Antithrombin Concentration Following Intravenous Administration of Estrogens, Proc. Soc. Exper. Biol. & Med. **94:** 92, 1957.
25. McGovern, J. J., and Steinberg, A. G.: Antihemophilic Factor Deficiency in the Female, J. Lab. & Clin. Med. **51:** 386, 1958.

THE USE OF CLINICAL DEXTRAN IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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RECENT studies have emphasized that properdin is a necessary component in the hemolytic system observed in paroxysmal nocturnal hemoglobinuria (P.N.H.).[1] The abnormal red cells in this disorder will hemolyze in serum containing magnesium, complement, and properdin more completely at a pH of 6.5 to 7.0. Pillemer and his associates[2, 3] have defined the role of properdin in immune reactions, and have demonstrated that many polysaccharides will interact with and bind properdin. In fact, its binding by zymosan has been part of the procedure for estimating serum properdin levels.

During a hemolytic crisis in a patient (A. A.) with P.N.H. who was undergoing cholecystectomy, clinical dextran was used to maintain the blood pressure until adequate blood replacement could be obtained. After the dextran infusion, the patient's physician observed a marked decrease in hemoglobinemia† which prompted further studies with a resultant preliminary comment.[4] Stratton and associates[5] have also discussed the use of clinical dextran in patients with P.N.H. Recently Crosby and Benjamin[6] have made certain in vitro studies concerning the effect of dextran on P.N.H. hemolysis.

The following clinical and laboratory studies were undertaken to ascertain whether clinical 6 per cent dextran infusions would inhibit hemolysis and decrease hemoglobinuria in patients with P.N.H. Certain in vitro observations have been attempted to define the function of dextran in the P.N.H. hemolytic system.

MATERIALS AND METHODS

Three men with P.N.H. were studied.‡ For the most part, clinical observations were made on an ambulatory basis, and at frequent intervals notes of illness or activities were recorded to correlate with hematologic measurements. Patients were briefly hospitalized for transfusions or special study. Two of them collected daily, separated, 12

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†We are indebted to Dr. Walter Cervoni for this observation.

‡We are indebted to Drs. W. B. Castle and C. P. Emerson, Jr., for allowing us to study patients under their care.

hour urine specimens (7:00 A.M. to 7:00 P.M.) for periods of 6 to 18 months by aliquoting samples from calibrated plastic collection bottles. These aliquots were used to measure urine hemoglobin concentration.

Plasma and urinary hemoglobin estimations were made by the benzidine method of Crosby and Furth.[7] Plasma and urine dextran levels were measured by the technique of Roe,[8] whereas serum complement was determined by the 50 per cent hemolysis procedure.[9] Serum properdin levels in one study were determined by Dr. Louis Pillemer.

Two types of clinical 6 per cent dextran solutions were used: the American type* with a mean molecular weight (M.M.W.) of 75,000, and a British preparation,† M.M.W. 152,000. In vitro studies were made with powdered dextran, M.M.W. 75,000, as used in preparations of the American type dextran.

Whole blood from patients with P.N.H. was collected in acid citrate dextrose (ACD) anticoagulant solution using sterile equipment. After centrifugation, the red blood cells were washed thrice in physiologic saline and stored in an approximately 30 per cent concentration in saline solution containing 0.0005M $MgCl_2$. The red cell suspensions were kept at 4° C. and were usually used for no longer than 1 week.

Serum from clotted blood was obtained from normal volunteers and from polycythemic donors who required phlebotomy. It was used immediately or stored in small aliquots at -65° C. In certain instances divalent cations were removed from serum by passing serum at 4° C. to maintain complement activity through Amberlite IRC50 resin in the sodium cycle.‡ This serum preparation did not allow P.N.H. red cell hemolysis in the absence of magnesium. Two solutions of purified human properdin were used for in vitro studies. One solution was prepared with the zymosan technique[2] and by assay contained 300 units per milliliter (HP-71§). The other solution was prepared by ethanol fractionation[10] and contained 600 units per milliliter (HP-FPSF‖). In some experiments rat serum was used as a source of properdin with an assumed properdin concentration of 50 units per milliliter. Magnesium and calcium were removed by passing chilled rat serum through an Amberlite IRC50 resin column. Thereafter the serum was adsorbed with 1:1 mixtures of human red cells of blood groups A, B, and O at 4° C. to remove heterologous antibodies. In the absence of cations, inactive complement allowed rat serum to be adsorbed without hemolysis. The sera were then retested with red cells at 37° C. to determine whether any residual hemolysins or agglutinins were present. If no hemolysis occurred, the serum was judged satisfactory for in vitro studies.

In vitro hemolytic studies were measured by methods similar to the technique originally described by Dacie and Richardson.[11] One milliliter aliquots of serum were acidified with 0.05 ml. of 3N HCl, to bring the pH to 6.5 to 6.6. Special additives to the system did not dilute the serum volume by more than 10 per cent and were added before the introduction of red cells. Five tenths milliliter of a 20 per cent red cell suspension was added and the mixture was layered with mineral oil and incubated 30 minutes in a 37° C. water bath. The mixture was immediately resuspended and centrifuged rapidly. The supernatant was measured for hemolysis in the spectrophotometer at 540 mμ; the per cent hemolysis was calculated using as a control a sample of red cells and serum that had been frozen and thawed to hemolyze all of the red cells, thus representing 100 per cent hemolysis.

CLINICAL OBSERVATIONS

The first opportunity for evaluating the clinical effect of dextran infusions occurred after a hemolytic reaction in a patient (M. P.) with P.N.H. who was given fresh plasma which contained Cr^{51}-labeled platelets[12] so their lifespan

*Powdered dextran, obtained from Dr. A. E. Osterberg, Abbott Laboratories, North Chicago, Ill.

†Intradex, supplied by J. T. Marsh, Glaxo Laboratories, Ltd., Greenford, England.

‡Obtained from Rohm & Haas, Philadelphia, Pa.

§Supplied by Dr. C. F. Hinz, Western Reserve University, Cleveland, Ohio.

‖Supplied by Dr. B. E. Sanders, Merck Institute, West Point, Pa.

might be observed. This transfusion accentuated the hemoglobinuria and hemoglobinemia. After the hemolytic reaction the patient received 500 ml. of 6 per cent dextran (Abbott) intravenously with no complications. Thereafter, for 3 consecutive days, 1,000 ml. of dextran was infused with a progressive decline of hemoglobinemia and hemoglobinuria (Fig. 1). Since hemoglobinuria often disappears after a hemolytic transfusion reaction, the patient was observed during the subsequent week. A second dextran infusion was given with similar results.

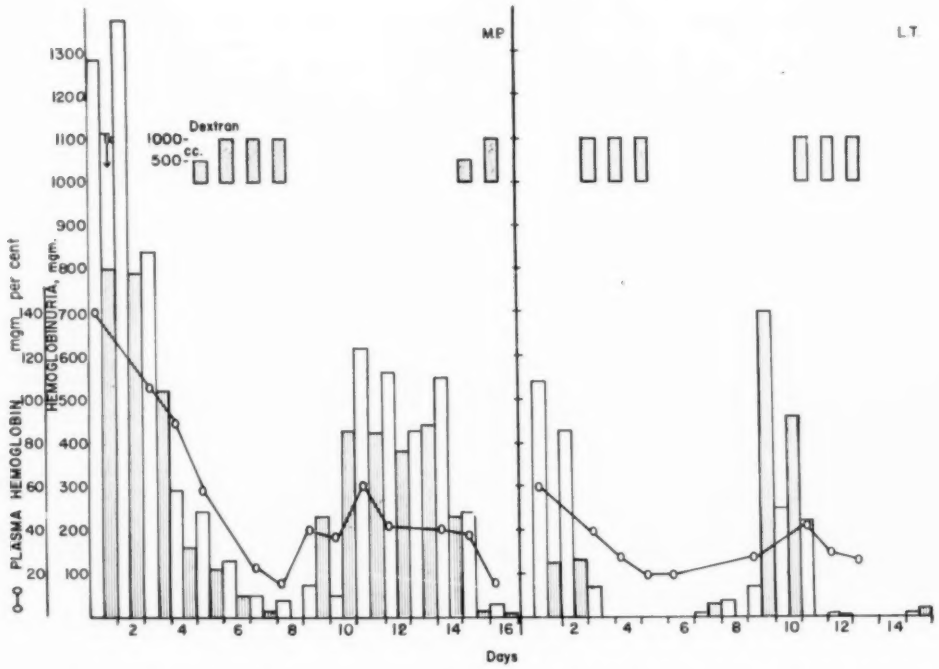

Fig. 1.—Patients M. P. and L. T. Changes in hemoglobinemia and hemoglobinuria after infusions of 6 per cent dextran. Daily urine specimens divided into daytime (open bar) and nocturnal (shaded bar) 12 hour samples. Initial hemolysis in Patient M. P. related to transfusion (Tx) of fresh plasma. The increased nocturnal hemoglobinuria in Patient L. T. after initial dextran infusions was compatible with the daily fluctuations in his pattern of hemoglobinuria. Patient L. T. has a more prolonged response to dextran infusion.

A second patient (L. T.) was given 1,000 ml. of 6 per cent dextran solution for 3 consecutive days to determine whether this clinical response could be duplicated. Here, the decrease in hemoglobinuria was more pronounced and prolonged than noted in the first patient (Fig. 1). A second period of dextran infusions followed the initial study to exclude any variability in hemoglobinuria.

These initial clinical observations suggested that P.N.H. hemolysis could be partially controlled by dextran infusions. To evaluate the practical application of dextran administration in patients with this disorder, a weekly therapy program was planned to determine whether the transient decreased hemolysis would lessen transfusion requirements. Patient M. P. received 9 weekly 1 L. infusions of 6 per cent dextran solution (M.M.W. 152,000). At the end of this period

his prolonged bleeding time, accompanied by epistaxis and gingival bleeding, indicated that continued dextran administration was not a safe procedure. During this interval his transfusion requirements did not decrease. The effect of dextran infusions on nocturnal hemoglobinuria is plotted in Fig. 2, and a similar period at a later date is plotted for comparison. After each infusion there was a lessening of hemoglobinuria. When dextran was given concurrently

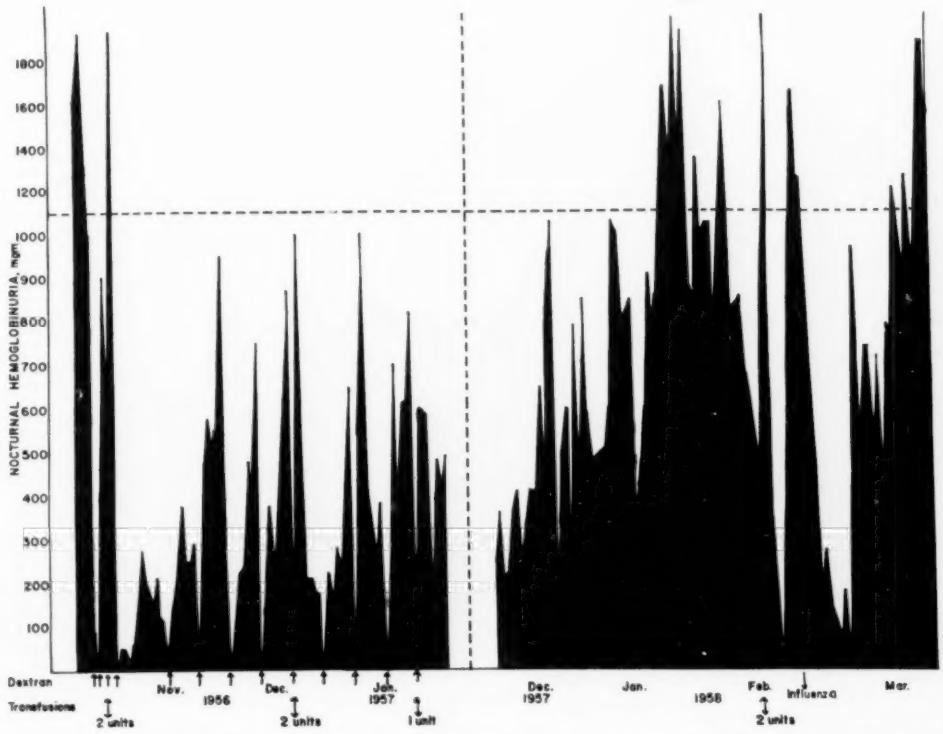

Fig. 2.—Patient M. P. Daily 12 hour nocturnal hemoglobinuria values plotted as shaded area during period of weekly 1 L. dextran infusions. Each infusion was followed by a decrease in nocturnal hemoglobinuria. To the right a comparable period 1 year later emphasizes that the normal variations of nocturnal hemoglobinuria do not decline to the lower levels noted following dextran infusions. An interval of decreased hemoglobinuria after 2 unit blood transfusion and after a 10 day episode of influenza are the only 2 instances to compare favorably with the decreased hemolysis following dextran.

with sedimented red cell transfusions, hemoglobinuria was more pronounced but less than with red cells alone. The intensity of hemoglobinuria decreased during the period of weekly dextran infusions as compared to the same interval 1 year later.

Each liter of dextran administered caused a rapid elevation of the plasma dextran level (Fig. 3). By the ninth week a plasma dextran concentration was achieved that would inhibit hemolysis (see In Vitro Observations). However, hemorrhagic complications prevented maintenance of such levels by means of which changes in the peripheral blood values could be appraised.

The daily fluctuations in hemoglobinuria emphasize the importance of reproducible observations in patients with this disorder (Fig. 2). Many factors

and agents affect hemoglobinuria[13] and the transient alterations in plasma volume related to dextran infusions may have been contributing factors in the inhibition of hemolysis. Two patients, M. P. and L. T., received 100 ml. of 25 per cent human serum albumin in 500 ml. of saline and 1,000 ml. of saline solution, respectively, to determine whether these infusions would alter hemoglobinuria. Two consecutive 1 L. 6 per cent dextran infusions are plotted in

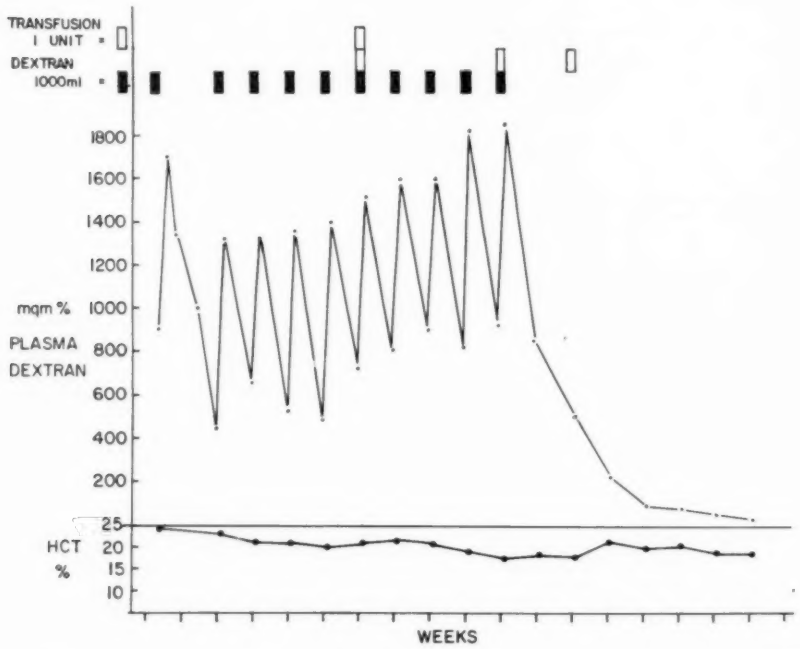

Fig. 3.—Patient M. P. Variations in plasma dextran levels after weekly infusions of 1 L. of 6 per cent British dextran solution. In vitro studies would indicate that an inadequate plasma dextran level was attained after 9 weeks to inhibit hemolysis.

Fig. 4 comparing the effects of human serum albumin and physiologic saline on nocturnal hemoglobinuria. After the albumin and saline infusions a transient decrease in hemoglobinuria was noted for several hours which in reality reflected a decreased hemoglobin concentration in a dilute urine. However, decreased nocturnal hemoglobinuria was noted only after dextran infusions.

Patients with P.N.H. have episodes of hemoglobinuria after transfusion.[14] Usually hemoglobinuria is less severe when the plasma is removed from the red cells which are in turn washed thrice in physiologic saline. However, such manipulations increase the risk of contamination. In several instances 2 patients with P.N.H. were given dextran infusions prior to transfusion of sedimented red cells.* Their alterations in hemoglobinuria are listed in Tables I and II. Comparative procedures of plasma removal and saline washed red cells are tabulated in these patients. Less hemoglobinuria was present when dextran

*After standing overnight at 4° C., whole blood collected in plastic bags with ACD anticoagulant may have 80 per cent of the plasma removed from the sedimented red cells.

was infused prior to transfusion. L. T. and M. P. experienced complete inhibition of hemolysis when adequate dextran was given prior to transfusions. The 3 patients studied have commented that the discomfort of transfusion (myalgia and lumbar back pain) was less severe when dextran was infused prior to transfusion. However, dextran had no influence on febrile reactions despite the absence of hemoglobinuria.

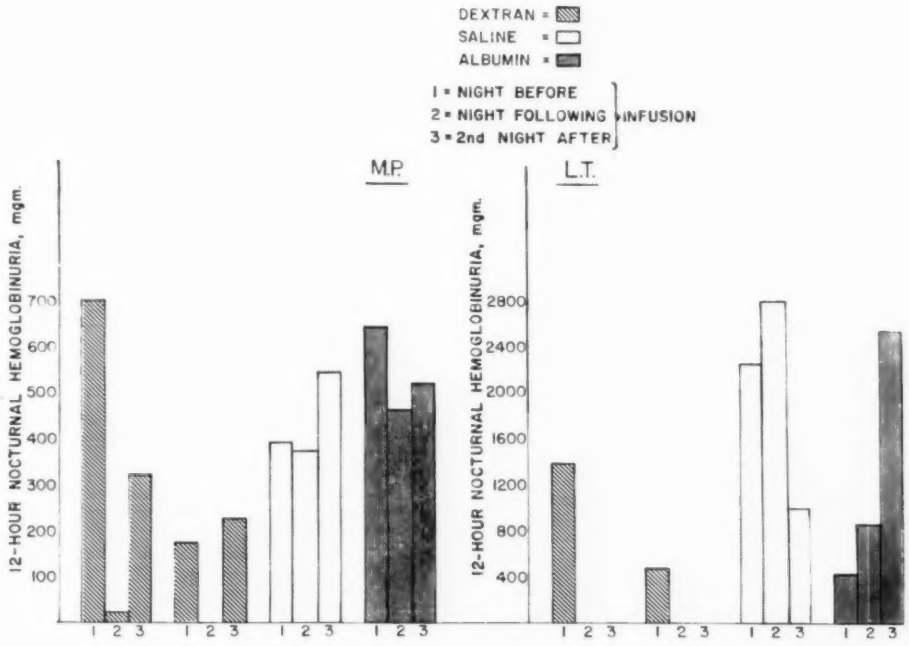

Fig. 4.—Patients M. P. and L. T. Levels of nocturnal hemoglobinuria after infusions of dextran, saline, and albumin as plasma expanders. Two infusions of dextran are plotted for each patient. There is no evidence that transient expansion of plasma volume by albumin or saline alters nocturnal hemoglobinuria.

IN VITRO OBSERVATIONS

The initial clinical observation prompted the following studies to determine how dextran inhibited hemolysis. The standard in vitro acidified hemolytic serum system was used with varying concentrations of dextran. Five to 25 mg. of dextran (M.M.W. 75,000) in 0.1 ml. of physiologic saline solution were added to 1.0 ml. of acidified serum. The per cent hemolytic inhibition is related to the number of red cells hemolyzed when 0.1 ml. of saline without dextran is added as the serum diluent. Less hemolysis was present as the dextran concentration was increased (Fig. 5). Patient M. P. had less inhibition with dextran than the other 2 patients. The maximum plasma dextran levels observed during the weekly infusions in M. P. (Fig. 3) did not reach a concentration that would inhibit hemolysis for any duration from the in vitro studies.

Hemolytic studies were performed to determine whether clinical dextran had any inhibitory effect on the components for hemolysis of P.N.H. red cells.

P.N.H. Red Cells.—Thrice washed red cells were suspended in 6 per cent clinical dextran (M.M.W. 75,000) solution and incubated at 37° C. for 30 minutes. The cells were then washed twice in physiologic saline solution and used in the standard acidified hemolytic serum system. The preliminary contact of dextran with the red cell membrane caused no inhibition of hemolysis.

Magnesium.—Utilizing serum devoid of divalent cations by exposure to Amberlite resin, $MgCl_2$ was added in progressive amounts to determine optimal concentration for hemolysis in the standard acidified serum system. A final concentration of 8 mm. provided maximal hemolysis with moderate inhibition at the level of 2 and 16 mm. It was postulated that any complexing of dextran with magnesium should allow enhanced hemolysis at 16 mm. compared to the inhibition previously noted. When varying amounts of dextran, from 5 to 25 mg., were added to 1 ml. serum containing 2 mm. and 16 mm. of magnesium, respectively, there was no alteration of the hemolytic inhibition noted from dextran. The 2 curves of hemolytic activity are parallel to indicate that dextran does not alter the function of magnesium.

TABLE I

TIME	HEMOGLOBINURIA (MG./24 HOURS)	THERAPY
I		
Day before Tx	580	
Day 1	280	1,000 ml. dextran + 2 units blood
Day 2	480	2 units blood
Day 3	1,170	2 units blood
II		
Day before Tx	2,825	
Day 1	870	1,000 ml. dextran + 2 units blood
Day 2	150	500 ml. dextran + 2 units blood
Day 3	50	
Day 4	0	500 ml. dextran + 2 units blood
Day 5	0	

Patient L. T. Two transfusion (Tx) studies with 6 per cent dextran infusions. One liter of dextran did not inhibit hemoglobinuria after transfusion of sedimented red cells: each unit derived from 500 ml. whole blood. When an additional 500 ml. of dextran was administered before additional transfusion in the second study, hemoglobinuria was abolished. During both procedures, the patient had febrile reactions with temperature rising to 104° F., possibly related to "buffy coat" sensitization.

Complement.—Since properdin is not necessary for isohemolysis of the ABO blood groups, this simple procedure was utilized to determine whether dextran interfered with complement dependent systems. The standard hemolytic system using 1.0 ml. of nonacidified serum with the same varying concentrations of clinical dextran, as described previously, was used. Hemolytic group O sera were mixed with group A cells. Sera were used in which hemolysis was not complete in order that inhibition might be observed. From these observations dextran (M.M.W. 75,000) in the concentrations which will inhibit P.N.H. hemolysis (20 to 25 mg. per milliliter) did not inhibit isohemolysins. Concentrations above 25 mg. per milliliter *did* inhibit hemolysis but such levels were not used for the in vitro studies, nor were they achieved in patients with P.N.H. after dextran infusions. Moreover, determination of the complement titer

using 1 ml. of serum with the same varying concentrations of clinical dextran, showed no significant difference between the control and those sera which contained dextran.

TABLE II

TIME	NOCTURNAL HEMOGLOBINURIA (MG./12 HOURS)	THERAPY	
I			
Day 2 before Tx	580		
Day 1 before Tx	600		
Day 1	433	2 units blood	Whole blood centrifuged
Day 2	925	2 units blood	to remove all plasma
Day 3	275		and "buffy coat" from
Day 4	50		red cell
II			
Day 2 before Tx	250		
Day 1 before Tx	220		
Day 1	320	2 units blood	
Day 2	350	2 units blood	Saline washed to remove "buffy coat"
III			
Day 2 before Tx	170		
Day 1 before Tx	230		
Day 1	340	2 units blood	Saline washed to remove
Day 2	730		"buffy coat"
IV			
Day 1 before Tx	860		
Day 1	490	500 ml. dextran + 2 units blood	
Day 2	525	500 ml. dextran + 2 units blood	
Day 3	270	500 ml. dextran + 1 unit blood	
Day 4	225		
V			
Day 2 before Tx	83		
Day 1 before Tx	104		
Day 1	0	1,000 ml. dextran + 2 units blood	

Patient M. P. Five transfusion (Tx) procedures. Alterations in nocturnal hemoglobinuria have been lessened by removing plasma and "buffy coat." Preliminary infusions of dextran will decrease the hemolysis further.

TABLE III

PATIENTS WITH P.N.H. RED CELLS	PROPERDIN SOURCE	PER CENT HEMOLYSIS IN 1.0 ML. ACIDIFIED SERUM (pH 6.6) WITH SALINE DILUENT = 100%		
		PROPERDIN 10 UNITS/ML. (%)	DEXTRAN (M.M.W. 75,000) 25 MG./ML. (%)	DEXTRAN 25 MG./ML. + PROPERDIN 10 UNITS/ML. (%)
M. P.	H.P.*	165	76	137
	R.S.†	174	--	151
L. T.	H.P.‡	268	74	264
	R.S.	164	74	122

Effect of properdin on P.N.H. red cell hemolysis. In each instance 1.0 ml. of acidified serum was used; the hemolysis observed with physiologic saline diluent as the 100 per cent value was utilized. Ten units of purified human or rat properdin activity enhanced P.N.H. hemolysis equally in patient M. P. The inhibitory effect of dextran almost was eliminated by the addition of 10 units of properdin.

*Purified human properdin (HP-71) 300 units per milliliter.

†Rat serum (assumed assay 50 units per milliliter).

‡Purified human properdin (HP-113-FPSF) 600 units per milliliter.

Properdin.—Serum properdin levels were only measured after clinical dextran infusions in 1 study. After administration of 500 ml. 6 per cent clinical dextran (M.M.W. 75,000) the preinfusion serum level of 6 units fell to less than 3 units immediately after the infusion was completed. The properdin value remained at 4 units for 3 days before returning to the pretreatment level.

Ten units of purified human properdin was added to the in vitro P.N.H. hemolytic serum system and augmented hemolysis as described by Hinz and co-workers.[1] The inhibition of hemolysis by dextran is eliminated by this amount of purified properdin. Similarly, absorbed rat serum will augment hemolysis and overcome the decreased hemolysis from dextran (Table III).

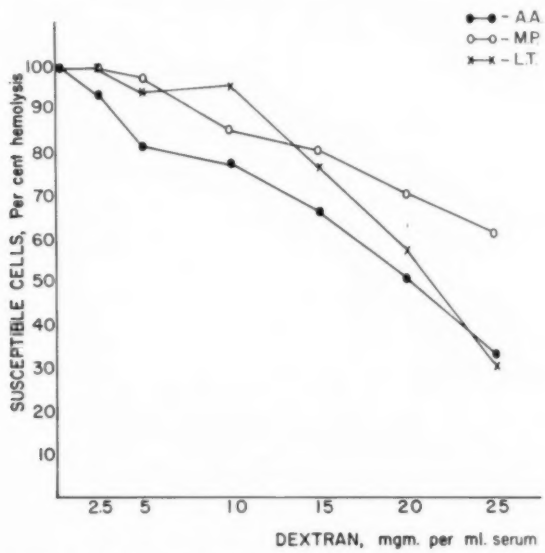

Fig. 5.—Inhibition of P.N.H. red cell hemolysis in 3 patients by dextran. Hemolysis noted in acidified serum pH 6.6, without added dextran plotted as the 100 per cent susceptible red cell value in each patient. Decreased hemolysis was observed with increased dextran concentration (M.M.W. 75,000).

Pillemer and co-workers[15] have emphasized that the low molecular weight clinical dextrans do not bind properdin, but, in some instances, high molecular weight dextrans will complex with properdin. When varying amounts of dextran of progressively higher molecular weights were added to the standard in vitro P.N.H. hemolytic serum system described, progressive hemolytic inhibition was noted (Fig. 6).* Dextrans with molecular weight above 300,000 have the same inhibition of hemolysis at 10 mg. per milliliter serum concentration as noted with the dextrans of lower molecular weight at 25 mg. per milliliter. Possibly the higher molecular weight fraction in the lower weight mixtures (in which mean molecular weight is measured) is the active component which inhibits hemolysis. These observations are suggestive evidence that clinical dextrans bind properdin in the P.N.H. hemolytic system.

*These dextrans were obtained by the National Research Council from the Commercial Solvents Corp., Terre Haute, Ind., through the courtesy of Dr. Margaret H. Sloan.

DISCUSSION

The in vivo and in vitro observations indicate that clinical dextrans will inhibit hemolysis in patients with P.N.H. To inhibit hemolysis, dextran solutions with a higher percentage of large molecular components seem desirable to maintain a dextran plasma level over a longer period of time. Over 80 per cent of the dextran molecular weight abundance of the American preparation is less than 100,000.[16] Since dextran molecules of less than 60,000 are rapidly excreted, clinical infusions of American type dextran will have 70 per cent of the plasma dextran eliminated in 24 hours. In most of the in vivo studies, British dextran solution has been used since the bulk of the molecular units

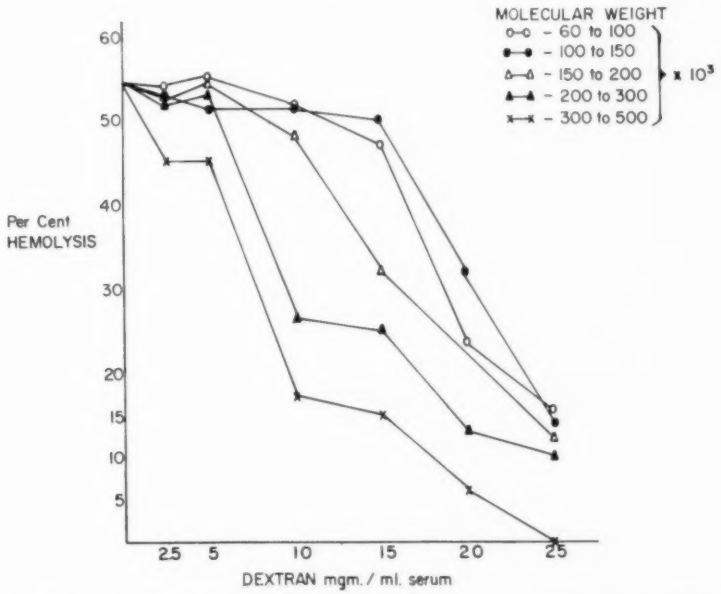

Fig. 6.—Patient L. T. Effect of dextrans of different mean molecular weights on hemolysis. Significant inhibition is only found at lower dextran concentration when molecular weight is above 200,000.

are above 100,000 and only 25 per cent is excreted in 24 hours.[17] The in vitro studies indicate that the larger molecular weight fractions inhibit hemolysis more completely. Both types of clinical infusions will suppress hemoglobinuria only transiently.

Previous reports have documented the hemorrhagic complications of dextran infusions.[18, 19] These reactions are related to the plasma dextran concentration. For the most part, they may be related to the higher molecular weight fractions that would persist in the plasma for more than 12 hours.[20] No bleeding complications were noted in the short term in vivo studies reported herein. However, after the repeated weekly 1 L. infusions over a period of 9 weeks, changes in capillary permeability were present in 1 patient. The dextran molecules that remained in his circulation from week to week represented large components that were excluded from the renal threshold. This patient, M. P.,

had a splenectomy several years prior to this study, and his blood platelet values were normal throughout the 9 week study and may have delayed the appearance of a prolonged bleeding time. Patients with thrombocytopenia are more sensitive to the hemorrhagic complications of dextran infusions.[19] Many patients with P.N.H. have a mild to moderate thrombocytopenia and, in such instances, dextran infusions should be followed carefully by frequent determination of the bleeding time. While the hemorrhagic complications exclude frequent infusions of dextran, consecutive daily 1 L. infusions of 6 per cent clinical dextran can be used for several days to inhibit hemolysis.

The responses of the 2 patients charted in Fig. 1 emphasize the variations to be expected in patients with P.N.H. Patient L. T. had a longer period free from hemoglobinuria than did Patient M. P. There was no evidence to relate these changes to variations in "susceptible red cells," for these observations have been repeated many times; L. T. always showed a more effective response to dextran. As noted above, M. P. had a serum properdin level of 6 units per milliliter, whereas L. T. had a serum properdin level of 3 units per milliliter. The difference in response to infusions in vivo and the more complete inhibition to higher concentrations of dextran in vitro in L. T. (Fig. 5) may be related to less circulating plasma properdin and more complete binding by dextran.

No untoward reactions have been observed in these 3 adult patients after frequent infusions of British and American type dextrans during the past 3 years. Several months have elapsed between infusions without any evidence of immune reactions. Adequate preparation with dextran infusions before transfusion will markedly inhibit or eliminate hemoglobinemia. The patients tolerate transfusions more comfortably and the laborious process of washing red cells with saline to remove plasma is not required. Dextran has not eliminated the pyrexia associated with transfusions of sedimented red cells to patients with P.N.H. They have received multiple transfusions, and the febrile reactions that persist have been attributed to an immune response to the "buffy coat" components.[21]

Does dextran combine with properdin to inhibit hemolysis of the P.N.H. red cell? The inability to demonstrate changes in serum properdin concentrations in vitro after mixing serum with various clinical dextrans does not support this hypothesis.[15] In one clinical study reported here properdin levels were measured after a dextran infusion. Although the 50 per cent decrease in properdin units after infusion is suggestive evidence for properdin binding by dextran, repeated observations are necessary. Purified human properdin will overcome the hemolytic inhibition of dextran in vitro (Table III). Finally, the increased hemolytic inhibition related to increased molecular weight of dextran may be compatible with the observations that high molecular weight "native" dextrans can complex with properdin.[15] The properdin assay measurement is dependent on the complexing of properdin with zymosan. Active properdin can be dissociated from bacterial wall polysaccharides after its initial removal from the serum.[22] It is reasonable to postulate that properdin may be inactivated in the P.N.H. hemolytic system by dextran only to be more firmly

complexed by zymosan in the assay procedure. Properdin bound to the larger molecular dextran components would remain inactive to explain the initial decrease in serum properdin levels noted in Patient M. P. after the infusion of dextran. This concept also has been proposed recently by Crosby and Benjamin.[6]

Lasch and associates[23] have emphasized that careful titration of heparin infusions will control hemolysis in patients with P.N.H. Although Pillemer and co-workers[15] found no evidence of properdin-heparin complexes, the affinity for zymosan in the titration system may have prevented proper interpretation. Previously, Crosby[24] had emphasized that heparin would inhibit P.N.H. hemolysis. These studies suggested that the in vitro effect was related to decreased complement activity. Recently, Fritsche and Martin[25] demonstrated decreased properdin units in the P.N.H. hemolytic system as the heparin concentration was increased. Hemolysis decreased as properdin values declined despite adequate serum complement levels. These studies expand the possible variety of polysaccharides that may bind or alter the function of properdin.

The P.N.H. red cell, magnesium concentration, and complement units have not been influenced by the concentrations of dextran used in the in vitro studies described. Other serum factors may be affected by dextran that are as yet undescribed. However, for a working hypothesis, properdin complexing by dextrans in vivo is an adequate explanation for the clinical effectiveness of these clinical polysaccharides.

SUMMARY

Clinical dextran is an effective agent for inhibiting the hemolytic mechanism in the patient with paroxysmal nocturnal hemoglobinuria (P.N.H.). Long-term administration is associated with hemorrhagic complications. At the present time 6 per cent clinical dextran infusions are of value to temporarily control hemolysis associated with infection, trauma, and transfusion reactions.

In vitro observations demonstrate that hemolytic inhibition is related to plasma concentration and molecular weight of the dextran solution. Evidence has been presented to demonstrate that dextran does not interfere with the red cell membrane, magnesium, or complement. It is proposed that dextran binds properdin to inhibit hemolysis.

REFERENCES

1. Hinz, C. F., Jordan, W. S., and Pillemer, L.: The Properdin System and Immunity. IV. The Hemolysis of Erythrocytes From Patients With Paroxysmal Nocturnal Hemoglobinuria, J. Clin. Invest. **35:** 453, 1956.
2. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C.: The Properdin System and Immunity. I. Demonstration and Isolation of a New Serum Protein, Properdin and Its Role in Immune Phenomena, Science **120:** 279, 1954.
3. Pillemer, L., Landy, M., and Shear, M. J.: Properdin System and Immunity. VII. Alterations in Properdin Levels and Resistance to Infection in Mice Following Administration of Tissue Polysaccharides, J. Exper. Med. **106:** 99, 1957.
4. Gardner, F. H.: Comments in: Discussion on Paroxysmal Nocturnal Hemoglobinuria, American Society of Hematology, April 7, 1957, Boston, Mass., Blood **13:** 93, 1958.
5. Stratton, F., Wilkinson, J. F., and Israels, M. C. G.: Clinical Dextran for Acute Episodes in Paroxysmal Nocturnal Hemoglobinuria, Lancet **1:** 831, 1958.

6. Crosby, W. H., and Benjamin, N. R.: Paroxysmal Nocturnal Hemoglobinuria: The Effect of Dextran on Hemolysis in Vitro, Acta haemat. **19:** 143, 1958.
7. Crosby, W. H., and Furth, F. W.: A Modification of the Benzidine Method of Hemoglobin in Plasma and Urine, Blood **11:** 380, 1956.
8. Roe, J. H.: The Determination of Dextran in Blood and Urine With Anthrone Reagent, J. Biol. Chem. **208:** 889, 1954.
9. Kabat, E. A., and Mayer, N. M.: Experimental Immunochemistry, Springfield, Ill., 1948, Charles C Thomas.
10. Spicer, D. S., Priester, L. I., Smith, E. V. C., and Sanders, B. E.: Properdin. Preparation From Fraction I, Fed. Proc. **17:** 313, 1958.
11. Dacie, J. V., and Richardson, N.: The Influence of pH on in Vitro Hemolysis in Nocturnal Hemoglobinuria, J. Path. & Bact. **55:** 375, 1943.
12. Aas, K. A., and Gardner, F. H.: Survival of Blood Platelets Labeled With Chromium,[51] J. Clin. Invest. **37:** 1257, 1958.
13. Crosby, W. H.: Paroxysmal Nocturnal Hemoglobinuria. Relation of the Clinical Manfestation to Underlying Pathogenic Mechanisms, Blood **8:** 769, 1953.
14. Dacie, J. V.: Transfusion of Saline Washed Red Cells in Nocturnal Hemoglobinuria, Clin. Sc. **7:** 65, 1948.
15. Pillemer, L., Schoenberg, M. D., Blum, L., and Wurz, L.: Properdin System and Immunity. II. Interaction of Properdin System With Polysaccharides, Science **122:** 545, 1955.
16. Squire, J. R., Bull, J. P., Maycock, W. D., and Ricketts, C. R.: Dextran: Its Properties and Use in Medicine, Springfield, Ill., 1955, Charles C Thomas.
17. Lockwood, A. R., and James, A. E.: Observations on the Molecular Weight Distribution of Dextran Preparations and Their Physiological Effects, Journées Transfusionnelles Geneve, pp. 9-15, 1953.
18. Carbone, J. V., Furth, F. W., Scott, R., and Crosby, W. H.: An Hemostatic Defect Associated With Dextran Infusion, Proc. Soc. Exper. Biol. & Med. **85:** 101, 1954.
19. Adelson, E., Crosby, W. H., and Roeder, W. H.: Further Studies of a Hemostatic Defect Caused by Intravenous Dextran, J. LAB. & CLIN. MED. **45:** 441, 1955.
20. Howard, J. M., Teng, C. T., and Loeffler, R. K.: Studies of Dextrans of Various Molecular Size, Ann. Surg. **148:** 369, 1956.
21. Brittingham, T. E.: Immunologic Studies on Leukocytes, Vox. Sanguinis, Basel **2:** 242, 1957.
22. Isliker, H. C.: The Properdin System, Vox. Sanguinis, Basel **1:** 8, 1956.
23. Lasch, H. G., Linke, A., and Sessner, H. H.: Zur Pathogenese und Therapie du paroxysmalen nächtlichen Hämoglobinurie, Acta haemat. **13:** 366, 1955.
24. Crosby, W. H., and Dameshek, W.: Paroxysmal Nocturnal Hemoglobinuria. The Mechanism of Hemolysis and Its Relation to the Coagulation Mechanism, Blood **5:** 822, 1950.
25. Fritsche, W., and Martin, H.: Properdin und Hämolyse; zur Hemmung der Hämolyse der Erythrocytes von Krauchen mit paroxysmalen nächtlichen Hämoglobinurie durch Heparin, Klin. Wchnschr. **35:** 1166, 1957.

LABORATORY METHODS

THE ISOLATION OF BLOOD GLUCOSE AS POTASSIUM GLUCONATE

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BETHESDA, MD.

THE availability of C^{14}-labeled glucose has resulted in investigations of the kinetics of glucose metabolism in rats,[1] rabbits,[2] dogs,[3] and humans.[4] In these studies, after the injection of the radioactive glucose, frequent sampling of the body glucose pool and determination of the glucose specific activity are required. The method commonly used for isolating glucose from the body pool is the precipitation of the phenylglucosazone derivative from protein-free filtrates of whole blood[1] or plasma[4] after addition of carrier glucose. This method for preparing the osazone of glucose in pure form, however, has many undesirable features. These have recently been enumerated by Berson and associates.[2]

Preparation of the glucosazone is a very tedious process which requires several hours. Moreover, it is difficult to obtain an osazone with the melting point characteristic for this compound. Because of this, many recrystallizations are necessary and frequently the osazones fail to precipitate after 2 or 3 recrystallizations. Over-all recoveries of glucose are therefore low, a fact of utmost consideration when dealing with the administration of small amounts of radioactivity to normal human subjects. Another drawback of the glucosazone derivative is that its chemical structure complicates degradative procedures whereby individual carbon atoms are isolated and their radioactivity is assayed.

Steele and co-workers[5] have attempted to obviate some of these difficulties by converting the glucosazone to the phenylosotriazole derivative. The over-all yield, however, was only 23 per cent of the theoretic yield. Berson and associates[2] resorted to counting plasma C^{14} activity directly without isolation of glucose. Hiatt[6] developed an elaborate method for isolation of blood glucose which employed resin column adsorption of glucose-6-phosphate formed enzymatically from glucose. Dunn and co-workers[7] devised a method whereby glucose carbon was isolated from blood by periodic acid treatment of blood filtrates with subsequent oxidation of the formic acid derived from carbons 1 to 5 of glucose to CO_2 and trapping of carbon 6 as the dimedon derivative. In the latter method neither crystalline glucose nor a chemically pure derivative of glucose was isolated.

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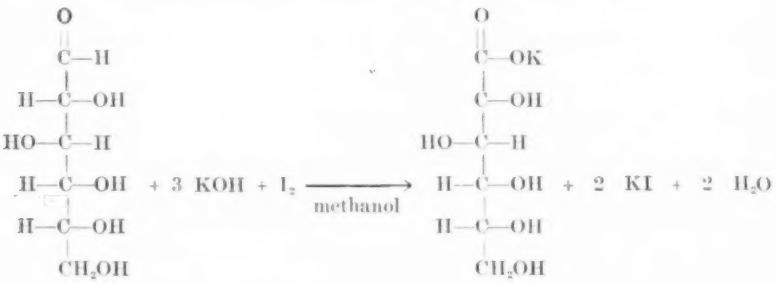
The limitations imposed by the previously existing methods prompted us to find a derivative of glucose easily isolated in pure state in high yield which can be chemically degraded with relative ease. By application of the hypoiodite oxidation of aldoses described by Moore and Link,[8] it has been possible to isolate blood or plasma glucose as potassium gluconate, a derivative which has the desired characteristics. The reaction carried out is:



METHOD

Reagents: (1) Glucose, (2) 90 per cent methanol, (3) absolute methanol, acetone free, (4) resublimed iodine, 280 mg. per 100 mg. of glucose to be oxidized, the required amount dissolved in 4 ml. absolute methanol, and (5) 4 per cent KOH in absolute methanol, 5.5 ml. per 100 mg. glucose to be oxidized.

Procedure.—A weighed amount of carrier glucose is added to a Somogyi[9] prepared protein-free filtrate of whole blood or plasma whose glucose content had previously been determined.* The filtrate is evaporated to dryness *in vacuo* at 40° C. Careful extraction of the residue is carried out with three successive 10 ml. portions of hot 90 per cent methanol, and the extracts are transferred to a 40 ml. centrifuge tube. Particulate matter is centrifuged down; the supernatant which contains the glucose is decanted to another 40 ml. centrifuge tube and evaporated to a thick syrup at 40° C. under a stream of nitrogen.

Two-tenths milliliter of water followed by 1.2 ml. absolute methanol are added; the sides of the tube are washed carefully with these additions. The mixture is stirred with a small rod to ensure complete solution of the syrup. A small amount of precipitate may again appear and is spun down.

The supernatant is transferred with a capillary pipette to a 25 ml. Erlenmeyer flask. The calculated amount of iodine dissolved in 4 ml. absolute methanol is added to the flask and the mixture is heated to 40° C. in a water bath. KOH in methanol is added dropwise with constant stirring. Upon addition of approximately half of the estimated volume of KOH solution a sticky yellow precipitate begins to form. With continued KOH addition and continuous stirring, the precipitate, potassium gluconate, becomes whitish and crystalline. The flask is removed from the water bath and allowed to stand at room temperature for 30 to 40 minutes for complete precipitation.

The gluconate is filtered by suction on a small Hirsch funnel, washed with absolute methanol, and dried on the funnel with acetone. Recrystallization is carried out by dissolving the product in a minimal amount of water, usually about 1 ml. Thirty milliliters of hot absolute methanol is added followed by addition of ethyl ether until a slight cloudiness appears. The flask is then set in ice for 20 to 30 minutes. The resultant gluconate is filtered, washed, and dried on the funnel with acetone as previously.

*The anticoagulant used in this laboratory is a sodium fluoride, calcium oxalate mixture 3:1 by weight, 45 mg. per 5 ml. of blood. Glucose determinations are performed by the glucose oxidase method employing the glucostat reagent of Worthington Co., Freehold, N. J.

Calculation.—Specific activity of the blood or plasma glucose is determined by multiplying observed specific activity of the isolated gluconate by a dilution factor which corrects for the presence of carrier glucose.

$$\text{S.A. } C^{14} \text{ Glucose (c.p.m./mM.)} = \text{S.A. Isolated Gluconate (c.p.m./mM.)} \left[\frac{\text{Mg carrier} + \text{Mg } C^{14} \text{ Glucose}}{\text{Mg } C^{14} \text{ Glucose}}\right]$$

RESULTS AND DISCUSSION

Moore and Link[8] originally described the iodine oxidation of aldoses to aldonic acids in the course of converting aldoses to their benzimidazole derivatives by coupling the aldonic acid with o-phenylenediamine. We have taken advantage of the fact that gluconate formed from glucose by the Moore and Link procedure is easily obtained in good yield as anhydrous crystalline needles.

By the capillary tube method, the crude gluconate formed in the reaction mixture was found to have a melting point, with decomposition, of 174° C. Pure potassium gluconate* melted at 178° C. One recrystallization from methanol was required to bring the melting point of gluconate prepared from blood filtrates to 177 to 178° C. Radioactive gluconate isolated from blood of individuals given C^{14}-labeled glucose was found to have a constant specific activity after 1 recrystallization. On the basis of the above criteria only 1 recrystallization of the gluconate is necessary in order to have a pure product.

Recoveries.—Because so little glucose is present in blood samples of the size ordinarily taken from animals or humans, one must resort to the addition of carrier glucose to each blood filtrate. Routinely, filtrates from 25 ml. of human whole blood have been used, and 100 mg. of carrier glucose has been added. Forty-four glucose isolations have been carried out in this laboratory. The yield of crude gluconate from the reaction mixture has averaged 73 per cent of theoretic. About 77 per cent of this material is recovered on recrystallization which makes an over-all yield of 56 per cent of the theoretic yield.

TABLE I. RECOVERY OF GLUCOSE ADDED TO SOMOGYI FILTRATES OF 5 ML. OF BLOOD

SAMPLE	ADDED GLUCOSE (MG.)	TOTAL GLUCOSE (MG.)	THEORETIC YIELD GLUCONATE (MG.)	GLUCONATE RECOVERED: REACTION MIXTURE (MG.)	GLUCONATE RECOVERED: ONE RECRYSTALLIZATION (MG.)	RECOVERY (%)
1	26.8	28.5	37.1	25.3	18.2	49.1
2	50.4	52.0	67.6	54.8	39.0	57.7
3	76.5	78.0	101.4	83.7	76.7	75.6

In order to ascertain whether the method is feasible and recoveries are high when smaller amounts of glucose are used, an experiment was performed in which approximately 25, 50, and 75 mg. of glucose were added to filtrates from 5 ml. aliquots of a single sample of whole blood. The results are shown in Table I. Over-all yields did not differ significantly from the average of 54 per cent obtained with 100 mg. glucose additions. The size of blood samples used depends on the quantity of radioactivity injected. The quantity of the carrier depends on the investigator's estimate of the ease of handling of various quantities of gluconate for his particular purposes.

*We wish to thank Dr. Frank Eisenberg for a gift of authentic potassium gluconate.

Starting with pure glucose in solution, Moore and Link found recoveries of crude gluconate of 90 per cent of the theoretic. We have similarly obtained 90 per cent recoveries. Possibly the 73 per cent recovery of crude gluconate reported here falls short of the 90 per cent expected because the hot methanol extraction is not entirely quantitative. Another more likely explanation is that salts in the filtrates acting as impurities decrease the yield. Because of impurities, the isolated crude gluconate may on rare occasions become hygroscopic on the filter. Such samples are immediately dissolved in water and recrystallized. No recrystallized sample has been observed to become hygroscopic.

Interference by Other Sugars.—Potassium salts of other aldonic acids may precipitate from alcoholic solution. With injection of C^{14}-labeled glucose in tracer quantities there is no danger of contamination of gluconate by other aldonates. Occasionally, one may be interested in the isolation and assay for radioactivity of glucose after the injection of trace quantities of other C^{14}-labeled sugars. Table II shows the results of an experiment in which minute amounts of radioactive galactose, ribose, fructose, and sorbitol were added to

TABLE II. CONTAMINATION OF GLUCONATE FORMED FROM UNLABELED GLUCOSE IN THE PRESENCE OF OTHER RADIOACTIVE SUGARS

SUGAR	AMOUNT ADDED TO GLUCOSE* (γ)	APPARENT GLUCOSE SP. ACT. (C.P.M./MM.)	APPARENT GLUCOSE SP. ACT. C.P.M./MM. AFTER GLUCONATE FORMATION RECRYSTALLIZATIONS			CONTAMINATION (%)
			1	2	3	
Galactose	2.1	25,580	19,230	19,230		75
Ribose	11.3	26,250		4,550	3,750	14
Fructose	1.8	26,000	408	360	246	0.9
Sorbitol	10.8	31,580	7,008	1,572	558	1.8

*150 mg.

glucose which was then converted to gluconate. In a tracer experiment in a human, a few milligrams of highly labeled sugar is injected, and would be distributed in body fluids so that only several gamma are found in a small blood sample. Therefore, the gamma added was negligible, but contained considerable radioactivity. It can be seen that recrystallization failed to separate the galactonate, and that ribonate formed in the reaction was only partially separated. On the other hand, the gluconate was almost entirely freed of fructose and sorbitol radioactivity. These results suggest that glucose isolation as gluconate may be feasible in the presence of certain sugars but that the amount of contamination must be determined experimentally for each individual sugar.

A similar experiment has been carried out in which a glucose precursor, pyruvate-2-C^{14}, was added to the unlabeled glucose. After 2 recrystallizations of gluconate approximately 4 per cent contamination was observed. The quantity of pyruvate added was 0.45 γ. Thus the contamination represents less than 0.02 γ of coprecipitation. These contamination experiments indicate the extreme sensitivity of radiochemical measurement, for by chemical criteria the gluconate isolated after addition of other radioactive substances would be considered pure.

Applications.—The osazone derivative has usually been plated directly for counting or combusted to carbon dioxide which may be counted in several ways.

Gluconate may be handled in a similar fashion; however, it is possible to degrade the gluconate so that certain carbon atoms may be isolated and radioassayed individually.

Periodate oxidation of gluconate by the method of Eisenberg[10] liberates carbon one as CO_2, carbons 2 to 5 as formic acid and carbon 6 as formaldehyde. The latter may be isolated from the reaction mixture as the dimedon derivative.[7] Formic acid can be oxidized to CO_2. Should this oxidation be carried out in the cold, C-1 and C-2 form glyoxylic acid, which can be isolated.[11] Knowing C-1 radioactivity permits calculation of the C^{14} in C-2 by difference.

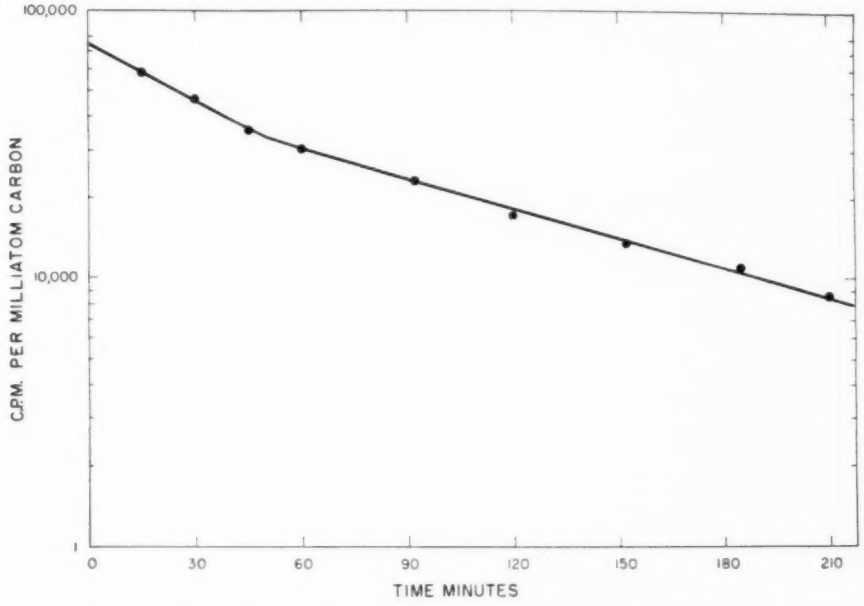


Fig. 1.—Change of specific activity of carbon 1 of blood glucose with time after the injection of 5 μc glucose-1-C^{14} into a normal human subject.

This treatment of gluconate permits performance of metabolic studies with glucose whose radioactivity is in a single carbon rather than uniformly distributed. Fig. 1 demonstrates the change in specific activity of carbon one of blood glucose with time after the intravenous injection of 5 μc of glucose-1-C^{14} to a normal subject. All of the counting was performed by liquid scintillation techniques, counting carbon 1 as CO_2 trapped in hyamine base.[12] Studies similar to this and others to determine the kinetics of carbon 6 metabolism after injection of glucose 6-C^{14} are being performed in this laboratory and will be the subject of a later publication.[13] The isolation of blood glucose as potassium gluconate provides a good starting material to conduct elaborate analysis of C^{14} glucose metabolism by analysis of the changes in radioactivity of individual carbon atoms.

SUMMARY

A method for the isolation of blood or plasma glucose as potassium gluconate is described. This derivative of glucose is superior to the commonly isolated

osazone derivative in that it is easily prepared in higher over-all yield. Moreover, the gluconate provides a good starting material for chemical degradation, thus facilitating the isolation of individual carbon atoms of the glucose molecule.

REFERENCES

1. Feller, D. H., Strisower, E. H., and Chaikoff, I. L.: Turnover and Oxidation of Body Glucose in Normal and Alloxan-Diabetic Rats, J. Biol. Chem. **187**: 571, 1950.
2. Berson, S. A., Weisenfeld, S., and Pascullo, M.: Utilization of Glucose in Normal and Diabetic Rabbits, Diabetes **8**: 116, 1959.
3. Steele, R., Altszuler, N., Wall, J. S., Dunn, A., and DeBodo, R. C.: Influence of Adrenalectomy on Glucose Turnover and Conversion to CO_2: Studies With C-14 Glucose in the Dog, Am. J. Physiol. **196**: 221, 1959.
4. Baker, N., Shreeve, W. W., Shipley, R. A., Incefy, G. E., and Miller, M.: C^{14} Studies in Carbohydrate Metabolism. I. The Oxidation of Glucose in Normal Human Subjects, J. Biol. Chem. **211**: 575, 1954.
5. Steele, R., Bernstein, W., and Bjerknes, C.: Single Phototube Liquid Scintillation Counting of an Easily Isolated Derivative of Blood Glucose, J. Appl. Physiol. **10**: 319, 1957.
6. Hiatt, H. H.: Studies of Ribose Metabolism. III. The Pathway of Ribose Carbon Conversion to Glucose in Man, J. Clin. Invest. **37**: 651, 1958.
7. Dunn, F., Friedmann, B., Maass, A. R., Reichard, G. A., and Weinhouse, S.: Effects of Insulin on Blood Glucose Entry and Removal Rates of Normal Dogs, J. Biol. Chem. **225**: 225, 1957.
8. Moore, S., and Link, K. P.: Carbohydrate Characterization, J. Biol. Chem. **133**: 293, 1940.
9. Somogyi, M.: Determination of Blood Sugar, J. Biol. Chem. **160**: 69, 1945.
10. Eisenberg, F.: Degradation of Isotopically Labeled Glucose via Periodate Oxidation of Gluconate, J. Am. Chem. Soc. **76**: 5152, 1954.
11. Eisenberg, F.: Personal communication.
12. Eisenberg, F.: Liquid Scintillation Counting, London, 1958, Pergamon Press, Inc., p. 123.
13. Segal, S., and Blair, A.: Studies in Glucose Metabolism. I. The Metabolism of Variously C-14 Labeled Glucose in Normal Man. In preparation.

MICRODETERMINATION OF GOLD IN BIOLOGIC FLUIDS EMPLOYING CHLORIC ACID AND ORTHOTOLIDINE

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VARIOUS colorimetric methods for the determination of gold have been proposed, employing p-diethylaminobenzylidine rhodanine,[1] malachite green,[2] orthodianisidine,[3-5] and orthotolidine (4, 4′-diamino-3, 3′-dimethylbiphenyl)[6, 7] reagents. Other procedures involved conversion to the orange colored bromaurate ion[8] and measurement of the color formed by the reduction of the metal to the colloidal form.[9-11] In most of these methods[1-8] the principle depends on (1) destruction and removal of interfering substances, (2) formation of gold chloride with aqua regia, and (3) oxidation of the various organic compounds and reagents by gold chloride to intensely colored products. In most cases the chromogenic solutions formed obey the Beer-Lambert law and serve as a basis for quantitation.

The orthodianisidine method of Block and Buchanan[5] has been used most extensively for determination of gold in tissue and biologic fluids. However, this method does not afford the sensitivity desired in the performance of refined metabolic studies in persons and animals. The excessive amounts of salts which result from digestion of samples of serum and urine interfere with formation of color. Inasmuch as the procedure of digestion by sulfuric acid and hydrogen peroxide is relatively tedious and requires much time, we undertook the development of a method which would have greater sensitivity, require a smaller volume of sample, lessen interference from excess of salts, and shorten the time required for digestion and formation of color.

MATERIALS AND METHODS

Chloric Acid Reagent.—Weigh into a 3 L. beaker 500 Gm. of potassium chlorate, add 900 ml. of distilled water, and heat until dissolved. Remove the mixture from heat and add 375 ml. of 72 per cent perchloric acid, stirring constantly. The covered beaker is kept in a refrigerator 12 to 24 hours to effect complete precipitation of the potassium perchlorate, then filtered with suction through Whatman 41 H filter paper. The filtrate contains approximately 28 per cent chloric acid.

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Standard Gold Stock Solution.—Dilute 10 mg. of gold sodium thiomalate,* which contains 5 mg. of gold per milliliter with distilled water to 50 ml. This solution contains 100 meg. of gold per milliliter† and is stable indefinitely when refrigerated.

Working Gold Standard.—Dilute 1 ml. of the stock solution to 10 ml. with water. Each milliliter contains 10 meg. of gold. This solution is prepared weekly.

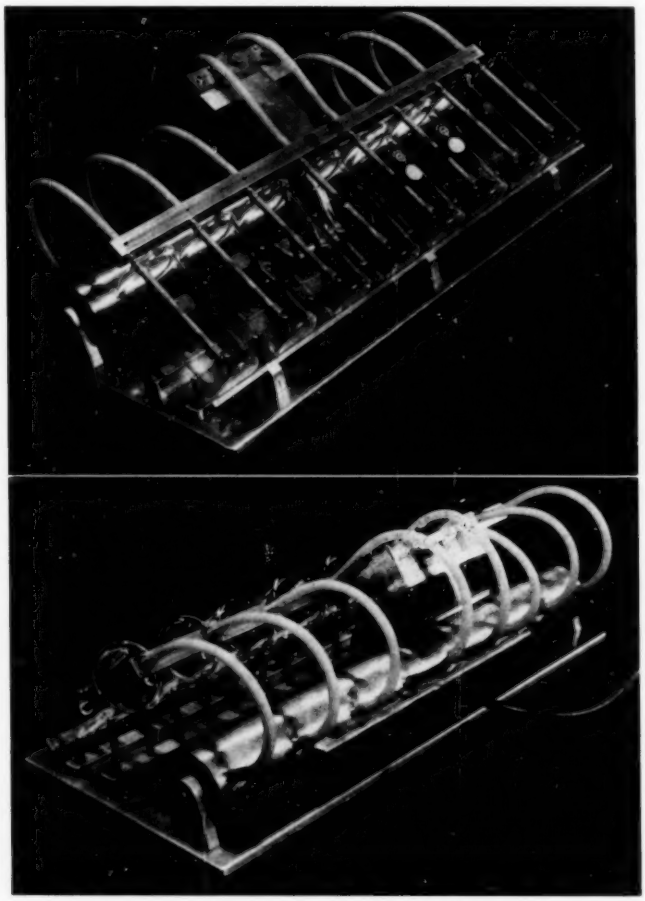

Fig. 1.—Two views of the apparatus devised to evolve aqua regia decomposition products in the determination of gold. Air introduced into the central chamber is distributed to mounted electrolytic beakers by means of mobile manifolds. The apparatus can be positioned so that the evolved air covers a maximum surface area.

Saturated Stock Solution of Orthotolidine.—Add 0.25 Gm. portions of *o*-tolidine to 150 ml. of boiling 2N H_2SO_4, with intermittent stirring, until a saturated solution is obtained. Boil the mixture for 5 minutes and filter while hot through glass wool to remove the insoluble and light, floating particles. Allow to crystallize by cooling to room temperature. Centrifuge

*Myochrysine, Merck Sharpe & Dohme, Philadelphia, Pa.

†This solution may be conveniently standardized by converting an aliquot to gold Chloride and by subsequent iodometric titration using a starch indicator with potassium iodide and sodium thiosulfate[11] according to the following reactions:

$$AuCl_3 + 2\ KI \rightleftharpoons AuCl + I_2 + 2\ KCL$$
$$I_2 + 2\ Na_2\ S_2\ O_3 \rightleftharpoons 2NaI + Na_2\ S_4O_6$$

at 1,500 r.p.m. for 5 minutes; decant and discard the supernatant. The material is recrystallized twice more and suspended in hot N H_2SO_4 until a supersaturated solution forms; this usually requires 1.5 to 2 L. of acid. The solution is allowed to stand 24 hours before use, at which time a layer of *o*-tolidine forms on the bottom of the container. This solution is stable indefinitely.

Working Solution of Orthotolidine.—Carefully remove 25 ml. of saturated *o*-tolidine stock solution from the saturated stock solution of *o*-tolidine without disturbing the precipitate and place in a 500 ml. volumetric flask. Add 40 ml. of 10N H_2SO_4, and a sufficient quantity of distilled water to bring the volume to the 500 ml. mark. This solution is made up weekly.

Bifluoride Reagent.—Place 0.75 Gm. of KHF in a 150 ml. stoppered polyethylene bottle and add 100 ml. of distilled water. This solution is prepared fresh daily.

Aqua Regia.—Three parts of concentrated reagent HCl is added to 1 part of concentrated reagent HNO_3. This solution is made daily.

Phenolphthalein Indicator.—One per cent solution in 95 per cent ethanol.

Ten per cent sodium chloride is used.

Eight per cent sodium hydroxide is used.

PROCEDURE

Digestion.—Pipette 1 to 3 ml. of human serum or plasma in a 250 ml. electrolytic beaker. Add 2 or 3 acid-washed Hengar crystals to the beakers with 7 ml. of chloric acid and 4 drops of 10 per cent NaCl. Cover the beaker with a borosilicate watch glass and digest on a hot plate until fumes of perchloric acid appear. At this point remove the watch glass and heat the crystalline residue until the dense white fumes of perchloric acid subside, but not to dryness. Remove the beaker from the hot plate and redissolve the crystalline residue in 5 ml. of distilled water, washing down the wall of the container. Allow the beaker to cool and neutralize the solution with 8 per cent sodium hydroxide using phenolphthalein as an indicator. Replace the beaker on a hot plate (temperature, 100 to 110° C.) and allow evaporation to proceed to the stage at which the crystalline salts just cover the bottom of the container. It is important not to dry the solution completely, since dehydration results in splashing and low recovery.

Formation of Gold Chloride.—Add 3 ml. of aqua regia to the cooled beaker; cover with a watch glass and allow to evaporate on the hot plate (temperature, 70 to 90° C.) to dryness. This may be observed by rotation of the flask on the cooler edge of the hot plate after the initial sign of formation of salt becomes evident. Remove the flask and, while still warm, introduce a slow stream of air (approximately 15 minutes) to evolve the nitrosyl chloride formed during decomposition of aqua regia. Replace the flask on the cooler edge of the hot plate and rotate to help evolve the last traces of nitrosyl chloride. Then introduce it under a stream of air for 45 minutes longer. An apparatus designed for this purpose is shown in Fig. 1.

Color Development.—Add 1 ml. of bifluoride solution to the beaker which contains the converted gold chloride; then add 10 ml. of working *o*-tolidine solution. The beaker is swirled to ensure thorough mixing. After 20 minutes the color is read in a suitable spectrophotometer at 427 mμ, or in a suitable colorimeter with a blue filter.

Standards.—Pipette 0.1, 0.2, and 0.4 ml. of working gold standards representing 1, 2, and 4 mcg. of gold into 250 ml. electrolytic beakers and proceed as previously. A gold-free serum or plasma blank is also carried through the determination.

Procedure for Gold Determination in Urine.—Introduce 10 to 50 ml. of measured urine in a 250 ml. electrolytic beaker, then add 5 or 6 acid-washed Hengar granules and 8 to 15 ml. of chloric acid. Place the beaker on a hot plate and allow digestion to proceed to the point at which the green yellow fumes of chlorine and lower chlorides disappear,

TABLE I. RECOVERY OF KNOWN AMOUNTS OF GOLD ADDED TO PLASMA, SERUM, AND URINE AS GOLD SODIUM THIOMALATE

MATERIAL USED	VOLUME	GOLD ADDED (MCG.)	GOLD FOUND (MCG.)	NO. OF SAMPLES
Serum and plasma	2 ml.	1.0	1.0	8
		1.0	0.9	3
		1.0	1.1	1
		1.0	1.2	1
		2.0	2.0	15
		2.0	1.8	3
		2.0	2.2	4
		2.0	2.1	1
		4.0	4.0	10
		4.0	3.9	6
		4.0	3.8	2
		4.0	4.1	2
		8.0	8.0	7
		8.0	7.8	6
		8.0	8.1	1
		8.0	8.2	2
Urine	10 ml.	2.0	2.0	2
		2.0	2.1	2
		2.0	1.9	2
		4.0	4.0	2
		4.0	4.2	1
		4.0	4.1	1
		4.0	3.9	1
		4.0	3.8	1
		8.0	8.0	1
		8.0	8.2	1
		8.0	8.1	1
		8.0	7.8	1

TABLE II. RECOVERY OF KNOWN AMOUNTS OF GOLD ADDED TO ENTIRE EXCISED RAT ORGANS AS GOLD SODIUM THIOMALATE

RAT ORGAN	GOLD ADDED (MCG.)	GOLD FOUND (MCG.)	NO. OF SAMPLES
Kidney	2.0	2.0	2
	2.0	2.2	2
	2.0	1.8	1
	4.0	4.0	1
	5.0	5.0	1
	5.0	4.8	1
	6.0	6.1	1
	8.0	8.0	3
	8.0	7.7	1
	10.0	10.0	1
	10.0	10.3	1
Spleen	2.0	2.0	1
	2.0	1.8	1
	5.0	5.2	1
	6.0	6.1	1
	6.0	5.9	1
Heart	4.0	4.0	1
	4.0	4.2	2
	4.0	3.8	1
	6.0	6.0	2
	8.0	7.9	1

Wet weights of kidneys were 0.7 to 1.3 Grams; of spleens, 0.4 to 0.7 Gram; of hearts, 0.3 to 1.4 Grams.

leaving a white crystalline mass on the bottom of the beaker. Do not allow the beakers to become completely dry. After cooling, neutralization with sodium hydroxide, conversion to gold chloride, and subsequent development of color are carried out as outlined. The colored solution is placed in a centrifuge tube and centrifuged at 2,000 r.p.m. for 5 minutes in order to remove the residual salts commonly present when larger volumes of urine are digested. Measurement of color is carried out as outlined.

Procedure for Gold Determination in Tissue and Feces.—Add 1 to 2 Gm. of wet tissue or 0.3 to 0.7 Gm. of dry tissue to 250 ml. electrolytic beakers and carry out digestion-neutralization conversion to gold chloride and subsequent color development as outlined. If, during digestion, dark brown fumes appear or the digest turns dark brown, remove the beaker immediately, add 5 ml. of chloric acid, and continue digestion.

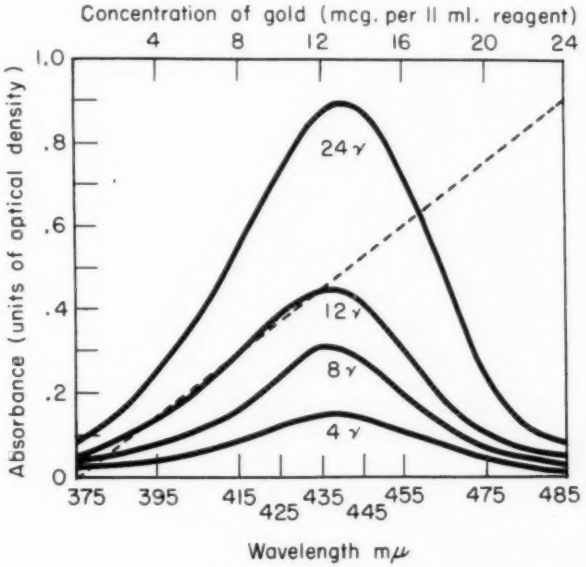

Fig. 2.—Absorption spectra of oxidized *o*-tolidine.

Standards.—Pipette 0.2, 0.4, and 0.8 ml. of working gold standard representing 2, 4, and 8 mcg. of gold into 250 ml. electrolytic beakers. Proceed as outlined under *Standards* for serum. A gold-free urine or tissue blank is carried through the entire procedure.

RESULTS

The results obtained by the addition of known quantities of auriothiomalate to serum plasma and urine are summarized in Tables I and II, and the number of samples analyzed and their distribution are indicated. The extinction coefficient for gold determination in biologic fluids using this method is 3.75×10^4.

DISCUSSION

The sensitivity of orthotolidine has been pointed out by Clabaugh[7] who was able to determine 1 to 4 gammas of gold in gold platings with an average deviation of 4 per cent. This reagent is currently used for determination of chlorine[12] in water. Chloric acid is used as a digestant in the determination in the serum of protein-bound iodine[13] and of total and phospholipid phosphorus.[14, 15]

The color obtained by oxidation of *o*-tolidine is maximal in 20 minutes and is stable up to 2 hours. It is not necessary to use freshly prepared reagents, since the *o*-tolidine working standard is stable for at least 1 week. In this laboratory the stock solution of *o*-tolidine has been found stable up to 7 months when kept at room temperature.

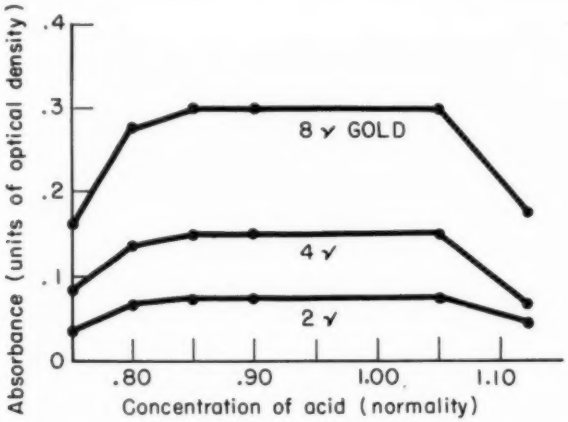

Fig. 3.—The stability of color formation of oxidized *o*-tolidine with varying concentrations of acid.

It is important that all chloric acid be completely destroyed during the digestion, so as not to interfere with development of color. Since the end product of chloric acid digestion is the formation of perchlorate salts, these salts do not pose a major problem. However, if the perchlorate salts are completely dried, loss of gold will occur.

It is also important to remove all of the nitrosyl chloride formed during the decomposition of aqua regia. This was accomplished by an air stream directed through rubber tubing connected so as to impinge on the bottom of an electrolytic beaker in the manner described by the evaporating apparatus shown in Fig. 1. The nitrosyl chloride may be detected by its characteristic odor.

Copper and iron[16] are two interfering substances commonly encountered in biologic fluids. Ferric iron in the amount of 5 mg. did not interfere with *o*-tolidine in the formation of color in the presence of 1 ml. of 0.75 per cent KHF. Copper, in a concentration of 1 mg. per 11 ml. solution, did not interfere with formation of color. Any chlorine, bromine, or nitrosyl chloride originally present must be removed. This was accomplished during digestion and the stage of air drying which followed conversion of gold to gold chloride by aqua regia.

Oxidized *o*-tolidine has an absorption peak at 437 mμ and obeys the Beer-Lambert law, when oxidation is caused by gold chloride in the range of 1 to 40 meg. of gold. (Fig. 2.) The color formed is stable over a wide range of concentration of acid (0.85N to 1.05N). (Fig. 3.) In an effort to determine the minimal amount of saturated *o*-tolidine required for development of color of a gold sample larger than ordinarily encountered in our studies (24 meg.), the

concentration of acid was kept at 0.85N and the saturated *o*-tolidine reagent was varied from 0.5 to 8 ml. The results (Fig. 4) indicate that the amount currently used is in excess of that needed for microdeterminations.

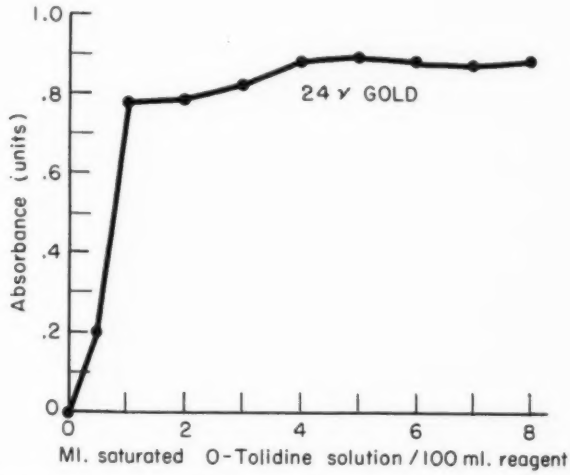

Fig. 4.—The intensity of color formation of oxidized *o*-tolidine with varying concentrations of *o*-tolidine.

Because of its instability, the use of gold chloride as a standard is expensive and impractical. Gold sodium thiomalate remains stable for long periods and may be routinely standardized, using the iodometric procedure described.

The principal advantages of this method are the relative ease of digestion with chloric acid, sensitivity, and stability of the color formed with *o*-tolidine.

SUMMARY

A method is described for determination of gold in biologic fluids using chloric acid digestion and subsequent formation of gold chloride, and oxidation of *o*-tolidine with gold chloride. The advantages of the method are ease of digestion, sensitivity, and stability of color formed.

We wish to acknowledge the cooperation of Dr. George Wilson of the Henry Ford Hospital, Rheumatism and Arthritis Unit, Detroit, Mich.

REFERENCES

1. Sandell, E. B.: Colorimetric Determination of Traces of Gold, Anal. Chem. **20**: 253, 1948.
2. Kulberg, C. M.: New Methods for Detecting and Determining Traces of Gold in the Presence of Copper, Zavodskaya Lab. **5**: 170, 1936.
3. Pollard, W. B.: Microdetermination of Gold, Analyst **62**: 597, 1937.
4. Jamieson, A. R., and Watson, R. S.: Determination of Minute Quantities of Gold in Urine, Analyst **63**: 702, 1938.
5. Block, W. D., and Buchanan, O. H.: The Microdetermination of Gold in Biological Fluids, J. Biol. Chem. **136**: 379, 1940.
6. Pollard, W. B.: The Use of Ortho-Tolidine as a Colorimetric Test for Gold, Analyst **44**: 94, 1919.
7. Clabaugh, W. S.: Method for Determining Small Amounts of Gold and Its Use in Ascertaining the Thickness of Electrodeposited Gold Castings, J. Res. Nat. Bur. Standards **36**: 119, 1946.

8. McBryde, W. A. E., and Yoe, J. H.: Colorimetric Determination of Gold as Bromaurate. Separation of Small Amounts of Gold by Solvent Extraction, Anal. Chem. **20**: 1094, 1948.
9. Fink, G. G., and Putnan, G. C.: Determination of Small Amounts of Gold With Stannous Chloride, Indust. Engen. Chem. (Anal. Ed.) **14**: 468, 1942.
10. Pierson, C. G.: Reducing Action of Mercurous Chloride; Separation Detection and Estimation of Arsenic, Gold, Platinum, Palladium, Tellurium and Iodine, Indust. Engin. Chem. (Anal. Ed.) **6**: 437, 1934.
11. Tukats, S.: An Iodometric Determination of Small Quantities of Gold in Animal Organs, Biochem. J. **260**: 143, 1933.
12. American Public Health Association: Standard Methods for the Examination of Water and Sewage, ed. 10, New York, 1955, American Public Health Association, Inc., pp. 66-71.
13. Zak, B., Willard, H. H., Myers, G. B., and Boyle, A. J.: Chloric Acid Method for the Determination of Protein-Bound Iodine, Anal. Chem. **24**: 1345, 1952.
14. Goodwin, J. F., Thibert, R., McCann, D., and Boyle, A. J.: Estimation of Serum Phospholipid and Total Phosphorus Using Chloric Acid, Anal. Chem. **30**: 1097, 1958.
15. Goodwin, J. F.: Total Phospholipid and Labile Phosphorus in Serum and Tissue Employing Chloric Acid and N-Phenyl-P-Phenylenediamine, Proc. Soc. Exper. Biol. & Med. **100**: 217, 1959.
16. Sandell, E. B.: Colorimetric Determination of Traces of Metals, ed. 2, New York, 1950, Interscience Publishers, Inc., p. 351.

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